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David W. Hibler, Ph.D.	May 29, 2007	SOM700/13004
Regarding:	Number of Pages	Hard Copy Follows
Patent Application Entitled " R(-) Desmethylselegiline and its Use to Treat Immune System Dysfunction"	70 (including cover page)	No
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Message:		

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Cheryl D. Blume, *et al.*

Serial No.: 10/790,658

Filed: March 1, 2004

For: R(-) DESMETHYLSELEGILINE AND
ITS USE TO TREAT IMMUNE
SYSTEM DYSFUNCTION

Group Art Unit: 1615

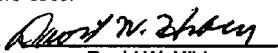
Appeal No.: 2007-1080

Examiner: L. Channavajjala

Atty. Dkt. No.: SOM700/4-4CIP2CON2DIV

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37 C.F.R. 1.6(d)

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May 29, 2007 
Date David W. Hibler

REQUEST FOR REHEARING UNDER 37 C.F.R. § 41.52

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants acknowledge the receipt of the Decision of the Board of Patent Appeals and Interferences ("the Board") for Vacatur and Remand ("the Decision") in the above-referenced case, mailed on March 26, 2007, which has been carefully reviewed and studied. Appellants respectfully request rehearing, as discussed in detail below.

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As set forth in 37 C.F.R. § 41.52(a)(1), the time limit for filing a request for rehearing is "within two months of the date of the original decision of the Board", or May 26, 2007, which falls on a Saturday and is therefore extended until Monday, May 28, 2007, which is a Federal holiday, and is therefore extended until Tuesday, May 29, 2007, under 37 C.F.R. § 41.52(a)(1), since the present communication is being transmitted by facsimile. Therefore, the present request for rehearing is timely filed, and Appellants believe no fees are due in connection with this request. However, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required in connection with the present request for rehearing, the Commissioner is hereby authorized to deduct the required fees from Vinson & Elkins Deposit Account No. 22-0365/SOM700/4-004CIP2CON2DIV.

REQUEST FOR REHEARING

Prior to the Decision, claims 26 and 34-62 stood rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement, and claims 26 and 34-62 stood rejected under 35 U.S.C. § 103(a) as obvious over Borbe *et al.* (*J. Neural. Transm. Suppl.* 32:131-137, 1990; "Borbe") in view of Barton *et al.* (*J. Neurooncol.* 1:333-346, 1983; "Barton") and Balsa *et al.* (*Biochem. Pharmacol.* 36:2723-2728, 1987; "Balsa"). A copy of the currently pending claims is attached as **Exhibit A** for the convenience of the Board.

In the Decision, the Board states "the appealed rejection under 35 U.S.C. § 112, first paragraph, is not in condition for a decision on appeal", because of "discordant results" reported in Billiau (*Adv. Immunol.* 62:61-130, 1996; "Billiau"), which Appellants cited in response to the rejection under 35 U.S.C. § 112, first paragraph. Also in the Decision, the Board states "that the appealed rejection under 35 U.S.C. § 103(a) does not rely on the most pertinent art", in that "Milgram [Milgram *et al.*, U.S. Patent No. 5,387,615] – cited in the specification (at 5: 14) – is

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closer and more relevant prior art than the references now of record", and therefore decided to "vacate the pending rejection under 35 U.S.C. § 103(a), and remand to the Examiner to determine whether a prior art rejection should be made over Milgram alone or in combination with other prior art", specifically, Borbe or Billiau. Appellants address each of the new grounds of rejection in turn.

A. Rejection of Claims 26 and 34-62 Under 35 U.S.C. § 112, First Paragraph

In support of Appellants' arguments rebutting the rejection of claims 26 and 34-62 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement, Appellants provided a publication by Billiau (*Adv. Immunol.* 62:61-130, 1996; "Billiau"). Billiau is a review article entitled "Interferon- γ : Biology and Role in Pathogenesis", which examines the role of decreased levels of interferon- γ in immune system dysfunction.

As set forth in Billiau, the cytokine IFN- γ plays a central role in the immune system, and immune dysfunction related to IFN- γ has been recognized in both immune deficiency and autoimmune diseases:

Medical interest in IFN- γ stems from awareness that a prominent target cell of IFN- γ , the macrophage, occupies a central position in the immune system. Adequate function of the IFN- γ /macrophage system is essential for natural as well as acquired resistance to infection and cancer. Malfunctioning of the system is recognized to be instrumental in inflammatory and autoimmune disease.

Billiau at page 62. In its review of Billiau, the Board states that "we find evidence in it, not addressed during prosecution, which raises questions about the adequacy of the enablement of the claimed invention" (the Decision at page 2). Specifically, the Board states that "(i)ncreased levels of gamma-interferon ... did not appear to always augment the immune system response" (the Decision at page 3), and provided two examples of such "discordant results". Appellants will address each of these examples in turn.

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In the first example, the Board states: "Billiau reports that gamma-interferon stimulated rather than inhibited HIV replication (Billiau at 96). This seems to suggest that increasing gamma-interferon levels to treat AIDS as recited in claims 37 and 60 would enhance the disease, rather than ameliorate it" (the Decision at page 3). The Board points to the following quote from Billiau: "In the case of HIV, activation of monocyteoid cells by IFN- γ was also found to stimulate rather than inhibit virus replication (292, 293)." Reference 292 is Biswas *et al.*, *J. Exp. Med.* 176:739-750, 1992 ("Biswas"; attached as **Exhibit B**), and reference 293 is Koyanagi *et al.*, *Science* 241:1673-1675, 1988 ("Koyanagi"; abstract attached as **Exhibit C**). Examination of these underlying references is necessary to give context to the Billiau quote cited by the Board.

Biswas reports that interferon- γ does induce the expression of HIV in persistently infected promonocytic cells (U1). But Biswas also points out that interferon- γ suppresses HIV replication in other cell lines, indicating that interferon- γ may play opposite roles in the regulation of HIV in different cell types:

In other studies, the combination of IFN- γ and TNF- α has been reported to induce cytolysis of HIV-infected cell lines but not of their uninfected counterparts, in association with a reduction of viral RNA (15). In addition, IFN- γ showed suppressive effects on HIV replication in certain T cell lines (16) and in the promonocytic cell line U937 (17, 18), which were acutely infected with HIV. In primary human monocyte-derived macrophages (MDM), IFN- α , - β , and - γ suppressed HIV replication when added to culture either before or up to 3 d after infection (19). However, other investigators have observed dichotomous effects of IFN- γ on virus replication in MDM, in that inhibition of virus expression was seen in cells pretreated with this lymphokine, whereas upregulation of p24 Ag production occurred if cells were treated with IFN- γ after infection (20). These studies suggest that IFN- γ may play different and even opposite roles in the regulation of HIV replication in different cell types.

Biswas at page 740, first column. Reference 20 cited above by Biswas is Koyanagi (cited as reference 293 in Billiau), and Biswas notes that the effects observed by Koyanagi are in

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disagreement with those observed by Kornbluth *et al.*, *J. Exp. Med.* 169:1137-1151, 1989 (reference 19 cited by Biswas in the above quote; attached as **Exhibit D**).

Biswas further states that "IFN- γ has been employed as a therapeutic agent ... in the treatment of HIV infection", noting that "reduction of plasma levels of p24 Ag, improvement of immune function, and clinical course have been described" (Biswas at page 748, second column), citing Heagy *et al.*, *J. Acquir. Immune Defic. Syndr.* 3:584-590, 1990; abstract attached as **Exhibit E**). Given the therapeutic use of interferon- γ to treat HIV infection, those of skill in the art would clearly understand that increasing interferon- γ levels would ameliorate AIDS in at least some patients.

In the second example, the Board states: "Billiau describes studies in which gamma-interferon enhanced tumor growth" (the Decision at page 3). But Billiau also describes how interferon- γ suppresses tumorigenicity, and that the observation of interferon- γ enhanced tumor growth is discordant with those results:

Rejection of some experimental tumors is associated with the presence of IFN- γ in the tumor tissue. That this IFN- γ contributes to this process is evident from studies with immunogenic autologous or isologous tumors, the rejection of which is abrogated by administration of neutralizing anti-IFN- γ antibodies (294, 295). Investigators have inserted the IFN- γ gene into nonimmunogenic murine tumor cells with high metastasizing potential and found that the IFN- γ secreting cells, when injected into syngeneic mice, had less ability than the parental cells to develop into tumors (296, 297); this suppression of tumorigenicity was reversed by the administration of anti-IFN- γ or anti-Lyt 2.2 antibodies. Discordant with these observations are several reports describing enhancement by IFN- γ of tumor growth or metastatic potential of experimental tumors.

Billiau at page 96, emphasis added.

Furthermore, in addition to the successful uses of interferon- γ in tumor therapy described in Billiau as shown above, cancer patients in a number of different clinical trials have responded positively to interferon- γ treatment. For example:

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1. one melanoma patient (out of eleven) had a 25% reduction in a cutaneous lesion in a phase I trial (Foon *et al.*, *Cancer Immunol. Immunother.* 20:193-197, 1985; abstract attached as **Exhibit F**);
2. a partial response of 8 weeks duration was observed in one patient (out of twenty) with hepatoma in a phase I trial (van der Burg *et al.*, *J. Biol. Response Mod.* 4:264-272, 1985; abstract attached as **Exhibit F**);
3. two patients (out of fifteen) with B-cell malignancies showed objective evidence of tumor regression during a phase I trial (Vadhan-Raj *et al.*, *Cancer Treat. Rep.* 70:6090614, 1986; abstract attached as **Exhibit F**);
4. one patient with metastatic melanoma had a complete response after two cycles of therapy, and another patient (out of thirty) entered partial remission after three cycles of therapy in a phase I/II study (Ernstoff *et al.*, *J. Clin. Oncol.* 5:1804-1810, 1987; abstract attached as **Exhibit F**);
5. 14.3% of renal cell cancer patients, 11.8% of multiple myeloma patients, 40% of chronic lymphocytic leukemia patients, 16.7% of non-Hodgkin lymphoma patients, and 67% of mycosis fungoides patients responded in a phase I/II trial (Kobayashi and Urabe, *Gan To Kagaku Ryoho.* 15:804-809, 1988; abstract attached as **Exhibit F**);
6. 31% of patients with metastatic renal cell carcinoma responded in a phase II trial, with the duration of response ranging from two months to over 44 months (Otto *et al.*, *Arzneimittelforschung* 38:1658-1660, 1988; abstract attached as **Exhibit F**);

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7. 30% of metastatic kidney cancer patients responded in a phase II trial, with the duration of response ranging from two months to over 34 months (Otto *et al.*, *Onkologie* 11:185-191, 1988; abstract attached as **Exhibit F**);
8. two complete responses and four partial responses (30%) were observed in a phase II trial with metastatic renal cell carcinoma (Aulitzky *et al.*, *J. Clin. Oncol.* 7:1875-1884, 1989; abstract attached as **Exhibit F**);
9. out of eight patients with advanced head and neck squamous cell carcinoma in a phase I/II trial, three had a clinically measurable response, four had stabilization of disease, and only one had progression while receiving treatment (Richtsmeier *et al.*, *Arch. Otolaryngol. Head Neck Surg.* 116:1271-1277, 1990; abstract attached as **Exhibit F**);
10. 36% of patients with metastasizing kidney carcinoma responded in phase II and III trials (Schneider *et al.*, *Helv. Chir. Acta* 57:415-421, 1990; abstract attached as **Exhibit F**);
11. 35% of patients with recurrent or persistent condylomata acuminata had complete clearance within 10 weeks of treatment, and no relapses occurred during the 16 week follow-up period (Reichel *et al.*, *Int. J. STD AIDS* 3:350-354, 1992; abstract attached as **Exhibit F**);
12. 15% of patients with metastatic renal cell carcinoma responded in a phase II trial of low dose interferon- γ (Ellerhorst *et al.*, *J. Urol.* 152:841-845, 1994; abstract attached as **Exhibit F**); and

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13. 32% of patients with residual ovarian cancer responded during a second-line treatment trial (Pujade-Lauraine *et al.*, *J. Clin. Oncol.* 14:343-350, 1996; abstract attached as **Exhibit F**).

Therefore, those of skill in the art would clearly understand that increasing interferon- γ levels would ameliorate tumor growth or progression in at least some cancer patients.

Appellants respectfully point out that the pending claims are drawn to methods of "treating a condition," not as a complete cure to the condition. This subject matter is analogous to that in *In re Sichert*, 566 F.2d 1154, 1160, 196 USPQ 209, 212 (CCPA 1977), in which the appeal court used the analogy of over-the-counter ointment drugs that have the purpose of stimulating blood circulation to distinguish treating from curing a condition. Use of an ointment to stimulate circulation and alleviate pain is not the same as treatment directed at curing the disease (arthritis) that has caused the condition. Furthermore, Appellants are not required to demonstrate "full treatment" of a disease prior to filing an application. As stated by the Federal Circuit in *Scott v. Finney* 32 USPQ2d 1115, 1120 (Fed. Cir. 1994):

Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.

Patentability of the pending claims does not require that the claimed methods be effective to treat any or all conditions produced by immune system dysfunction associated with reduced levels of γ -interferon production. As stated in the Manual of Patent Examining Procedure ("the MPEP"), § 2164.08(b):

The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would

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be inoperative or operative with expenditure of no more effort than is normally required in the art.

Appellants respectfully assert that the claims are enabled because it is well within the skill of one in the art to determine whether a condition produced by immune system dysfunction is associated with reduced levels of γ -interferon production, and whether administering the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ -interferon production. Measuring reduced levels or increased levels of γ -interferon in a mammal are well within the skill of one in the art, and do not require undue experimentation.

As stated in the specification, the ability of the R(-) enantiomer of desmethylselegiline to restore γ -interferon production supports the conclusion that R(-)-desmethylselegiline is able to treat a condition in a mammal produced by immune system dysfunction (see Example 11 of the specification, at pages 38-41). Given that the malfunctioning of the IFN- γ /macrophage system is recognized to be instrumental in inflammatory and autoimmune diseases, the ability of R(-)-desmethylselegiline to restore IFN- γ production will "bolster a patient's normal immunological defenses [and] be beneficial in the treatment of a wide variety of acute and chronic diseases including cancer, AIDS, and both bacterial and viral infections" (see the specification at page 38, lines 12-14).

Further, the specification (at least from page 9, line 8, to page 10, line 20) provides sufficient guidance to one of skill in the art for administering R(-)-desmethylselegiline to mammals for treating any or all of the disorders claimed. For example, at page 9, lines 8-21, the specification states:

The optimal daily dose of R(-)DMS [the R(-) enantiomer of desmethylselegiline], S(+)DMS [the S(+) enantiomer of desmethylselegiline], or of a combination, such as a racemic mixture, of R(-)DMS and S(+)DMS, useful for the purposes of the present invention is determined by methods known in the art, e.g., based on the severity of

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the disease or condition being treated, the condition of the subject to whom treatment is being given, the desired degree of therapeutic response, and the concomitant therapies being administered to the patient or animal. Ordinarily, however, the attending physician or veterinarian will administer an initial dose of at least about 0.015 mg/kg, calculated on the basis of the free secondary amine, with progressively higher doses being employed depending upon the route of administration and the subsequent response to the therapy... These guidelines further require that the actual dose be carefully titrated by the attending physician or veterinarian depending on the age, weight, clinical condition, and observed response of the individual patient or animal.

A person skilled in the art could readily determine the effective amount of R(-)-desmethylselegiline required to achieve a therapeutic effect based upon animal pharmacology and early phase clinical trials in humans, both of which are standard activities and practices in the pharmaceutical industry.

Based on the foregoing arguments, Appellants respectfully submit that the rejection of claims 26 and 34-62 under 35 U.S.C. § 112, first paragraph, has been overcome, and accordingly requests that the rejection be withdrawn or overruled.

B. Rejection of Claims 26 and 34-62 Under 35 U.S.C. § 103(a)

In the Decision, the Board states that "Milgram is more pertinent prior art than any of the references now cited by the Examiner under the § 103 rejection", and that "the Examiner should determine whether a § 103 rejection is appropriate in view of Milgram alone and/or combined with Borbe and Billiau" (the Decision bridging pages 3 and 4).

With regard to a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) in view of Milgram alone, the Decision states that "Milgram describes the use of L-deprenyl for treating immune system dysfunction", and that "L-deprenyl – also known as selegiline – is a selective monoamine oxidase B (MAO-B) inhibitor", but admits that "Milgram does not disclose the use of

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the claimed L-deprenyl metabolite, desmethyl-selegiline, to treat immune system dysfunction" (the Decision at page 4).

Appellants agree that "Milgram does not disclose the use of the claimed L-deprenyl metabolite, desmethyl-selegiline, to treat immune system dysfunction", and further point out that each of the pending claims actually recite use of the R(-) enantiomer of desmethylselegiline to treat immune system dysfunction. Furthermore, Milgram teaches dosages of between 0.1 mg/kg and 5.0 mg/kg of body weight of L-deprenyl, whereas claim 26 (and claims 34-39 and 42 dependent therefrom), claim 43 (and claims 44 and 45 dependent therefrom), and claim 57 recite a dosage of at least 0.015 mg/kg of the R(-) enantiomer of desmethylselegiline, which is between 6 and 7-fold lower than the dosage taught by Milgram. Since Milgram also teaches that "(i)t is important that the dosage [of L-deprenyl] be at levels expressed herein rather than at Parkinson's disease levels (up to 25X lower), otherwise no beneficial effects may be achieved" (Milgram in paragraph preceding Examples), Milgram does not teach or suggest the lower doses taught in certain aspects of the present claims.

Additionally, Milgram does not teach methods of treating immune system dysfunction that is associated with reduced levels of γ -interferon, or treatments that result in an increase of γ -interferon production, as recited in claim 26 (and claims dependent therefrom), claim 43 (and claims dependent therefrom), and claim 46 (and claims dependent therefrom), but, rather, treatments of immune system dysfunction that stimulate secretion of tumor necrosis factor- α , interleukin-6, or granulocyte-macrophage colony stimulating factor (see claim 1 of Milgram). Milgram also does not teach the treatment of immune system dysfunction caused by infectious disease (as recited in claims 35 and 58), treatment of cancer produced by immune system dysfunction (as recited in

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claims 38 and 61), or treatment of immune system dysfunction caused by cancer chemotherapy (as recited in claim 43 (and claims 44 and 45 dependent therefrom)).

In order to establish a *prima facie* case of obviousness, a reference (or combined references) must teach or suggest all of the claim limitations. Therefore, a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) in view of Milgram alone would clearly be improper, since Milgram does not teach or suggest the use of R(-)-desmethylselegiline to treat immune system dysfunction, certain dosages used to treat immune system dysfunction, the treatment of immune system dysfunction associated with reduced levels of γ -interferon, the treatment of immune system dysfunction caused by infectious disease, the treatment of cancer produced by immune system dysfunction, or the treatment of immune system dysfunction caused by cancer chemotherapy.

With regard to a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) in view of Milgram and Borbe, the Decision states that while “Milgram does not disclose the use of the claimed L-deprenyl metabolite, desmethyl-selegiline, to treat immune system dysfunction”, “Borbe (abstract; pp. 135-36) teaches that the MAO-B inhibitory activity of desmethyl-selegiline is ‘nearly equipotent to selegiline [L-deprenyl] after multiple oral administration’”, and that “(i)n view of Borbe’s teaching that the inhibitory activity of desmethyl-selegiline is ‘nearly equipotent’ to L-deprenyl, the Examiner should determine whether it would have been obvious to have utilized desmethyl-selegiline to treat immune system dysfunction” (the Decision at page 4). Appellants respectfully point out that in addition to all of the other deficiencies of Milgram discussed above, Borbe does not teach “that the inhibitory activity of desmethyl-selegiline is ‘nearly equipotent’ to L-deprenyl.” Rather, Borbe teaches that R(-)-desmethylselegiline retains no more than about one-third of the potency of selegiline *ex vivo*, which would lead those of skill in the art to expect that R(-)-desmethylselegiline, if therapeutically useful at all, would demonstrate no advantages over

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selegiline, and in fact would be less advantageous by requiring higher doses of the enantiomer to achieve the same inhibitory activity as selegiline.

Borbe specifically investigated the “*dose-response-relationship*” of “single oral doses of selegiline or [R(-)-desmethylselegiline],” as well as the “*restoration* of the MAO-B-inhibition … following a three-days oral treatment” with selegiline or R(-)-desmethylselegiline (Borbe at page 133, emphasis in original). The results of these experiments must not be read beyond the questions they were intended to answer, nor serve as the basis for impermissible hindsight reconstruction of the claimed subject matter (*In re Dow Chemical Co.*, 837 F.2d 469, 5 USPQ 2d 1529 (Fed. Cir. 1988)). To characterize the inhibitory action of selegiline and R(-)-desmethylselegiline, both compounds were orally administered to rats, and the half-maximal inhibitory dose (ID₅₀) for selegiline was 0.43 ± 0.02 mg/kg, and for R(-)-desmethylselegiline was 1.21 ± 0.06 mg/kg (Borbe at page 134, Table 1). Although Borbe characterized the activity of selegiline and R(-)-desmethylselegiline in this experiment as “nearly equipotent”, in fact, Borbe teaches that R(-)-desmethylselegiline is less potent than selegiline by a factor of three (Borbe at pages 133-134). In Borbe, the *in vitro* data, which showed that R(-)-desmethylselegiline was less active than selegiline by a factor of 60, was compared to the *ex vivo* data, which showed that R(-)-desmethylselegiline was less active than selegiline by a factor of three. After comparing the large difference in the *in vitro* and *ex vivo* potency of selegiline and R(-)-desmethylselegiline, Borbe nevertheless somehow concluded that the *ex vivo* potency of selegiline and R(-)-desmethylselegiline were “nearly equipotent”. But the data in Borbe clearly contradicts this conclusion.

Despite Borbe’s characterization of the *ex vivo* potency of R(-)-desmethylselegiline as “nearly equipotent” to selegiline, Borbe does not teach the equipotency of selegiline and R(-)-desmethylselegiline. Rather, Borbe teaches that R(-)-desmethylselegiline retains no more than

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about 33% of the potency of selegiline *ex vivo* (a factor of 3). Hence, persons skilled in the art would expect that the R(-) enantiomer of desmethylselegiline, if therapeutically useful at all, would demonstrate no advantages over selegiline, and in fact would be less advantageous than selegiline because R(-)-desmethylselegiline is one-third less potent. Persons skilled in the art would also have had the negative expectation that R(-)-desmethylselegiline would be relatively (*i.e.*, several fold) less therapeutically potent than selegiline, thus limiting or eliminating the potential usefulness of R(-)-desmethylselegiline as a pharmaceutical compound.

In contrast, Appellants have found that R(-)-desmethylselegiline has advantages over selegiline in a variety of therapeutic applications. All Borbe shows is that R(-)-desmethylselegiline is less effective than selegiline. Therefore, Borbe does not suggest, much less teach, that R(-)-desmethylselegiline would be equivalent to, let alone preferred to, selegiline to therapeutically treat a condition produced by immune system dysfunction.

The statement in the Decision that "Borbe (abstract; pp. 135-36) teaches that the MAO-B inhibitory activity of desmethyl-selegiline is 'nearly equipotent to selegiline [L-deprenyl] after multiple oral administration'" focuses on the Borbe teaching that selegiline inhibits MAO-B activity by $85.83 \pm 1.22\%$, whereas R(-)-desmethylselegiline inhibits MAO-B activity by $82.6 \pm 0.34\%$. But this data must be interpreted in context. These measurements were taken 24 hours after a course of treatment of one dose of 5.0 mg/kg selegiline or R(-)-desmethylselegiline per day for three days (multiple doses), to determine whether R(-)-desmethylselegiline, like selegiline, is an effective irreversible inhibitor of MAO-B after saturation of the system (Borbe at pages 134-135). The measurements in Table 2 (Borbe at page 136) led Borbe to conclude that R(-)-desmethylselegiline "is an irreversible blocker of MAO-B, nearly equipotent to selegiline after multiple oral administration" (Borbe at page 131).

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The purpose of this multiple dose experiment was to study the restoration of MAO-B activity after system saturation with an irreversible MAO-B inhibitor. Thus, the experiment was designed to effectively saturate the system of a rat to completely inhibit MAO-B-activity in rat brain. Borbe analyzed the measurements of Figure 1 as representing the reappearance of MAO-B-activity in a time-dependent fashion, which "might reflect the de-novo-synthesis of the enzyme" (Borbe at page 136). This experiment was not designed to compare the relative inhibitory activities of selegiline and R(-)-desmethylselegiline, but rather to inhibit all MAO-B activity in the subject, and then study the reappearance of MAO-B-activity. The previous *dose-response-relationship* experiment described above, which demonstrated that R(-)-desmethylselegiline is a factor 3 less potent than selegiline, is the data that a person skilled in the art would have examined to compare the relative inhibitory effects of selegiline and R(-)-desmethylselegiline.

Thus, against the negative expectations of a person skilled in the art (as taught by Borbe) that R(-)-desmethylselegiline is less potent than selegiline, Appellants discovered that R(-)-desmethylselegiline provides surprising and unexpected pharmacological effects. Even assuming, *arguendo*, that the therapeutic potential of R(-)-desmethylselegiline might somehow be deemed *prima facie* obvious over selegiline, a "*prima facie* case of obviousness based on structural similarity is rebuttable by proof that the claimed compounds possess unexpectedly advantageous or superior properties" (the MPEP, § 2144.09). Thus while Borbe taught that R(-)-desmethylselegiline was at least 3-fold less potent than selegiline, Appellants found that R(-)-desmethylselegiline has a potency equal to or greater than selegiline in a variety of therapeutic applications. This result would have been surprising to one of skill in the art based on Borbe.

As noted above, in order to establish a *prima facie* case of obviousness, the references when combined must teach or suggest all of the claim limitations. As admitted in the Decision, "Milgram

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does not disclose the use of the claimed L-deprenyl metabolite, desmethyl-selegiline, to treat immune system dysfunction”, and Borbe does not cure this deficiency of Milgram, since Borbe does not teach or suggest that “that the MAO-B inhibitory activity of desmethyl-selegiline is ‘nearly equipotent to selegiline [L-deprenyl] after multiple oral administration’”. Borbe also does not cure any of the other deficiencies of Milgram, as discussed in detail above, since Borbe does not teach or suggest certain of the claimed dosages used to treat immune system dysfunction, the treatment of immune system dysfunction associated with reduced levels of γ -interferon, the treatment of immune system dysfunction caused by infectious disease, the treatment of cancer produced by immune system dysfunction, or the treatment of immune system dysfunction caused by cancer chemotherapy. Therefore, a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) over Milgram in view of Borbe is also improper.

Finally, with regard to a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) in view of Milgram and Billiau, the Decision states that “Milgram also does not teach that L-deprenyl ‘leads to an increase in gamma-interferon production’ as recited in claim 26 and others”, but that “Billiau teaches that gamma-interferon is produced by T lymphocytes (pp. 63-66)”, and thus “the Examiner should determine whether it would have been expected that an improvement in T lymphocyte function, as taught by Milgram (Example 2, cols. 7-8) would also be associated with increased gamma-interferon levels” (the Decision bridging pages 5 and 6). Once again, however, Billiau does not cure the deficiency that “Milgram also does not teach that L-deprenyl ‘leads to an increase in gamma-interferon production’ as recited in claim 26 and others”, since Billiau does not teach or suggest R(-)-desmethylselegiline, let alone the use of R(-)-desmethylselegiline to increase γ -interferon production, as recited in each of the pending claims. Therefore, a rejection of claims 26 and 34-62 under 35 U.S.C. § 103(a) over Milgram in view of Billiau is also improper.

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C. Conclusion

In light of the foregoing remarks, Appellants respectfully request that the Board address the two new grounds of rejection raised in the Decision on the record, and withdraw or overrule the rejection of claims 26 and 34-62 under 35 U.S.C. § 112, first paragraph and 35 U.S.C. § 103(a).

Respectfully submitted,



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EXHIBIT A

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CLAIMS

1-25. (Cancelled)

26. (Previously Presented) A method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ -interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, at a daily dose, administered in a single or multiple dosage regimen, of at least about 0.015 mg, calculated on the basis of the free secondary amine, per kg of the mammal's body weight, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ -interferon production in the mammal.

27-33. (Cancelled)

34. (Previously Presented) The method of claim 26, wherein said R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.

35. (Previously Presented) The method of claim 26, wherein the condition produced by immune system dysfunction is caused by infectious disease.

36. (Previously Presented) The method of claim 26, wherein the immune system dysfunction is age-dependent.

37. (Previously Presented) The method of claim 26, wherein the condition produced by immune system dysfunction is AIDS.

38. (Previously Presented) The method of claim 26, wherein the condition produced by immune system dysfunction is cancer.

39. (Previously Presented) The method of claim 26, wherein the condition produced by immune system dysfunction is in response to a vaccine.

40. (Previously Presented) The method of claim 26, wherein the daily dose is between about 0.5 mg/kg and about 1.0 mg/kg.

41. (Previously Presented) The method of claim 26, wherein the daily dose is at least about 1.0 mg/kg.

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42. (Previously Presented) The method of claim 26, wherein the mammal is a human.
43. (Previously Presented) A method of treating a condition in a mammal produced by immune system dysfunction caused by cancer chemotherapy which is associated with reduced levels of γ -interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, at a daily dose, administered in a single or multiple dosage regimen, of at least about 0.015 mg, calculated on the basis of the free secondary amine, per kg of the mammal's body weight, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ -interferon production in the mammal.
44. (Previously Presented) The method of claim 43, wherein the R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.
45. (Previously Presented) The method of claim 43, wherein the mammal is a human.
46. (Previously Presented) A method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ -interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ -interferon production in the mammal.
47. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.
48. (Previously Presented) The method of claim 46, wherein the mammal is a human.
49. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered orally.

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50. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered non-orally.
51. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered parenterally.
52. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered transdermally.
53. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered buccally or sublingually.
54. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered intravenously.
55. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered subcutaneously.
56. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered intra-peritoneally.
57. (Previously Presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline is administered at a daily dose of at least about 0.015 mg/kg of the mammal's body weight, calculated on the basis of the free secondary amine.
58. (Previously Presented) The method of claim 46, wherein the condition produced by immune system dysfunction is caused by infectious disease.
59. (Previously Presented) The method of claim 46, wherein the immune system dysfunction is age-dependent.

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60. (Previously Presented) The method of claim 46, wherein the condition produced by immune system dysfunction is AIDS.
61. (Previously Presented) The method of claim 46, wherein the condition produced by immune system dysfunction is cancer.
62. (Previously Presented) The method of claim 46, wherein the condition produced by immune system dysfunction is in response to a vaccine.

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EXHIBIT B

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Interferon γ Induces the Expression of Human Immunodeficiency Virus in Persistently Infected Promonocytic Cells (U1) and Redirects the Production of Virions to Intracytoplasmic Vacuoles in Phorbol Myristate Acetate-differentiated U1 Cells

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Summary

Interferon γ (IFN- γ), a lymphokine that exerts multiple immunoregulatory effects, has been found to be elevated in the plasma, cerebrospinal fluid, and lymph nodes of human immunodeficiency virus (HIV)-infected individuals and has shown variable effects on HIV replication in acutely infected cells. In the present study, we have demonstrated that IFN- γ is a potent modulator of HIV expression in persistently infected U1 promonocytic cells in which virus production is characterized by a constitutive state of relative latency. Direct stimulation of U1 cells with IFN- γ (10–1,000 U/ml) activated HIV expression, as measured by reverse transcriptase (RT) activity in the culture supernatant and increased levels of cell-associated viral protein and mRNAs. These effects on virus expression were not accounted for by the induction of endogenous TNF- α secretion, as previously described in U1 cells stimulated with phorbol myristate acetate (PMA). At the ultrastructural level, the stimulatory activity of IFN- γ was correlated with HIV particle production in intracytoplasmic vacuoles along with the differentiation of U1 into macrophage-like cells. Furthermore, costimulation of U1 cells with IFN- γ and PMA significantly increased the accumulation of vacuole-associated HIV concomitant with decreasing membrane-associated particles and RT activity production, as compared with cells stimulated with PMA alone. No evidence of spontaneous secretion of intracellular vacuole-associated virus was obtained by kinetic analysis of the RT activity released in the supernatants throughout the culture period unless cells were deliberately disrupted. These findings suggest that vacuole-associated virions likely represent a relatively stable intracellular reservoir of HIV, as previously described in primary macrophages infected *in vitro* or in infected macrophages in the brains of patients with acquired immune deficiency syndrome. The reduced levels of RT activity observed in the culture supernatants of U1 cells stimulated with PMA in the presence of IFN- γ were not indicative of a suppressive effect of IFN- γ on PMA-induced expression of HIV proteins and mRNAs, either directly or mediated by the release of IFN- α/β . This study suggests that IFN- γ may play an important role as an inducer of HIV expression in infected mononuclear phagocytes.

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IFN- γ is a potent lymphokine produced by activated T cells that mediates several immunomodulatory and antimicrobial effects (reviewed in references 1–4). Both *in vivo* and *in vitro* studies have previously investigated the potential role of IFN- γ in HIV infection. Elevated levels of IFN- γ and/or of the IFN- γ -inducible factor neopterin have been found in the plasma

and cerebrospinal fluid (CSF)¹ of HIV-infected individuals at various stages of disease (5–7). In contrast, PBMC from HIV-

¹ Abbreviations used in this paper: CSF, cerebrospinal fluid; MDM, monocyte-derived macrophages; MP, mononuclear phagocytes; RT, reverse transcriptase.

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infected individuals are usually characterized by a significantly impaired ability to generate IFN- γ in vitro (8–10), although purified subpopulations of T cells showed an intact capacity to secrete this lymphokine (11). High levels of IFN- γ RNA were recently demonstrated in the lymph nodes of HIV-infected individuals (12), suggesting that this lymphokine may influence the ability of HIV to replicate in CD4 $^{+}$ T lymphocytes and mononuclear phagocytes (MP) in vivo.

In vitro, IFN- α and IFN- β , but not IFN- γ , inhibited HIV replication in mitogen-stimulated PBMC (13) in which IFN- γ may actually act as an endogenous autocrine/paracrine inducer of HIV replication in conjunction with TNF- α/β (14). In other studies, the combination of IFN- γ and TNF- α has been reported to induce cytolysis of HIV-infected cell lines but not of their uninfected counterparts, in association with a reduction of viral RNA (15). In addition, IFN- γ showed suppressive effects on HIV replication in certain T cell lines (16) and in the promonocytic cell line U937 (17, 18), which were acutely infected with HIV. In primary human monocyte-derived macrophages (MDM), IFN- α , - β , and - γ suppressed HIV replication when added to culture either before or up to 3 d after infection (19). However, other investigators have observed dichotomous effects of IFN- γ on virus replication in MDM, in that inhibition of virus expression was seen in cells pretreated with this lymphokine, whereas upregulation of p24 Ag production occurred if cells were treated with IFN- γ after infection (20). These studies suggest that IFN- γ may play different and even opposite roles in the regulation of HIV replication in different cell types. However, little or no information is available on the effects of IFN- γ in cells persistently infected with HIV. In this regard, several in vivo and in vitro studies have underscored the importance of persistent HIV infection in the pathogenesis of AIDS (21). Quantitative studies have demonstrated that in the peripheral blood compartment the number of infected cells that are in a state of either relative or absolute viral latency exceeds by about 10-fold the number of cells actively expressing HIV RNA or proteins (22). Most of these circulating infected cells are CD4 $^{+}$ T lymphocytes, whereas monocytes in the peripheral blood are infrequent targets of HIV infection in vivo (22–24). In contrast, terminally differentiated macrophages represent a major viral reservoir in several tissues and organs, including the brain (25, 26) and the lungs (27) of HIV-infected individuals, and have been found to express high levels of viral RNA (25, 26). One distinctive aspect of HIV infection in macrophages is the ability of the virus to bud from and accumulate into intracellular vacuolar compartments (28–32), an uncommon feature in infected T lymphocytes. This observation, coupled with the lower susceptibility of MP to the cytidal effects of HIV (23, 28), has suggested that tissue macrophages may represent important viral reservoirs that play a major role in the establishment of a state of chronic infection typical of most infected individuals (21, 32). Therefore, understanding whether physiologic host factors can influence the ability of HIV to assemble and accumulate in intracellular compartments as opposed to the plasma membrane of infected macrophages may be of importance for

designing therapeutic strategies targeting HIV at its different cellular and sub-cellular sites of expression. In this regard, it has been reported that IFN- α effectively suppressed the release of HIV particles from the plasma membrane of chronically infected T lymphocytic cells (33–35), a phenomenon that had been already described in cells persistently infected with other retroviruses after treatment with IFNs (36).

In the present study, we have investigated the effect of IFN- γ on the chronically infected promonocytic cell line U1, in which virus expression is modulated by several cytokines and pharmacologic agents (reviewed in references 21 and 37). PMA stimulation of U1 cells not only induces HIV production, but also induces the terminal differentiation of these cells along the mononuclear phagocytic lineage in association with the accumulation of HIV particles in Golgi-derived intracellular vacuoles (38), as described in primary infected macrophages (28–32). Thus, we determined the effects of INF- γ alone or in costimulation with PMA on cellular morphology, virus production, and virion compartmentalization in U1 cells.

Materials and Materials

Chronically HIV-infected Cell Lines. The U1 cell line was established from a population of U937 promonocytic cells surviving an acute infection with HIV-1, as described previously in detail (38). U1 cells contain two integrated copies of HIV proviral DNA, and are characterized by low levels of constitutive virus expression that can be modulated by several cytokines and pharmacologic agents (21, 37–40). 1–2 \times 10 5 U1 cells/ml were resuspended in RPMI 1640 (M.A. Whitaker Bioproducts, Walkersville, MD) containing 10% FCS, plated in 96-well flat-bottomed plates (Costar, Cambridge, MA), and incubated with either rIFN- γ (Genzyme, Boston, MA), rIFN- α_{2b} (Schering, Kenilworth, NJ), rIFN- β (kind gift of Dr. Sidney Petska, UMDNJ, Robert Wood Johnson Medical School, Piscataway, NJ), PMA (10 $^{-7}$ to 10 $^{-4}$ M) (Sigma Chemical Co., St. Louis, MO), rTNF- α , or rTNF- β (100 U/ml) (Genzyme), at 37°C in 5% CO₂. Supernatants from the cell cultures were collected at various times after stimulation and stored at -70°C until tested for the presence of reverse transcriptase (RT) activity.

RT Activity Assay. 5 μ l of culture supernatants was added in duplicate or triplicate to 25 μ l of a mixture containing poly(A), oligo(dT) (Pharmacia Fine Chemicals, Piscataway, NJ), MgCl₂, and [³²P]-labeled deoxythymidine 5'-triphosphate (dTTP) (Amersham Corp., Arlington Heights, IL), and incubated for 2 h at 37°C. 6 μ l of the mixture was spotted onto DE81 paper, air-dried, washed five times in 2 x SSC buffer, and two additional times in 95% ethanol. The paper was then dried, cut, and counted on a scintillation counter (LS 7000; Beckman Instruments Inc., Fullerton, CA). Variability of replicate cultures was always <15%.

Western Blot Analysis of Cell-associated HIV Proteins. Lysates were prepared from U1 cells either unstimulated or stimulated for 48 h with IFN- γ (1,000 U/ml), PMA (10 $^{-6}$ M), or costimulated with IFN- γ plus PMA. 20 μ l from the lysate of 10 7 cells was added to each lane and subjected to electrophoresis through 10–20% gradient polyacrylamide gels (Integration Sep. Sci., Hyde Park, MA) for 6 h. The migrated proteins were then transferred overnight onto nitrocellulose filters. After saturation with a 5% milk solution, filters were incubated for 2 h with 1:1,000 (vol/vol) dilution of an AIDS patient serum containing high titers of anti-HIV Ab recognizing most of the major viral proteins (33). Filters were then washed

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and incubated for 90 min with 125 I labeled protein A (200,000 dpm/ml), washed, air-dried, and exposed overnight to x-ray film.

Analysis of HIV RNA. U1 cells were either unstimulated, or stimulated for 24 h with IFN- γ (1,000 U/ml), TNF- α (100 U/ml), and PMA (10^{-8} M), or costimulated with PMA plus IFN- γ . Total RNA was extracted from 2×10^6 U1 cells by the guanidine thiocyanate phenol method using an RNA isolation kit (Stratagene, La Jolla, CA). In some experiments, different amounts of RNA were transferred to nitrocellulose filters in an RNA slot blot Minifold II apparatus (Schleicher & Schuell, Inc., Keene, NH), and the filters were hybridized overnight with a 32 P labeled 6.4-kb DNA probe (pBenn 5; map position, 1.7–8.1 kb), washed, and exposed to x-ray film as described (41). For Northern blot analysis, 10 μ g of total RNA extracted at different times after stimulation was loaded per lane on a 0.8% agarose formaldehyde gel and transferred to nitrocellulose. The filters were baked and hybridized for 12 h with a 32 P labeled HIV-LTR homologous probe (SST-BssHII). Filters were washed and exposed to x-ray film. The labeled probe was removed from the filters by washing at 80°C in 0.1 \times SSC containing 0.1% SDS; the filters were then rehybridized with a 32 P labeled β -actin cDNA probe, as previously described (42).

Ultrastructural Studies. U1 cells were stimulated with either IFN- α (20–100 U/ml), IFN- β (100 U/ml), IFN- γ (100–1,000 U/ml), PMA (10^{-8} M), or costimulated with IFN- α and PMA, IFN- β and PMA, or IFN- γ and PMA for 36–120 h. Cells were spun, fixed in glutaraldehyde, and then postfixed in OsO₄, dehydrated in graded ethanol and propylene oxide, and embedded in plastic, as described (33). For each sample, 100 consecutive cell sections were screened for the presence and location of HIV. Only sections at or close to the center of the cell and Golgi compartment (based on known average size of cell and nucleus) were included for evaluation. The findings were tallied as percentage of cells with HIV budding from or intimately associated with the plasma membrane only, HIV present in cytoplasmic vacuoles only, or HIV observed at both sites in the same cell.

Results

IFN- γ Induces HIV Expression in Chronically Infected U1 Cells. Stimulation of U1 cells with different concentrations of IFN- γ (10–1,000 U/ml) induced HIV production, as measured by RT activity in the culture supernatants, although to a lesser extent than did PMA stimulation (Fig. 1). Higher concentrations of IFN- γ (up to 5,000 U/ml) did not result in a significant increase of virus production as compared with the levels of induction observed with 500–1,000 U/ml of this lymphokine (data not shown). Thus, IFN- γ , which has been previously reported to induce HIV expression in acutely infected primary MDM (20), is another in the group of cytokines capable of inducing virus expression in persistently infected monocyte cells (reviewed in reference 37). In this regard, cytokines that stimulate HIV expression may induce de novo transcription of viral RNAs, as demonstrated for TNF- α and TNF- β (21, 37, 39–42), or may predominantly affect post-transcriptional steps in the HIV life cycle, as shown for IL-6 (42). We therefore investigated the effect of IFN- γ on the expression of HIV RNA in U1 cells. Unstimulated U1 cells showed detectable levels of fully spliced 2-kb mRNA of the regulatory genes *tat*, *nef*, and *rev* (Fig. 2), as previously reported (40). Stimulation of U1 cells with IFN- γ resulted in the ac-

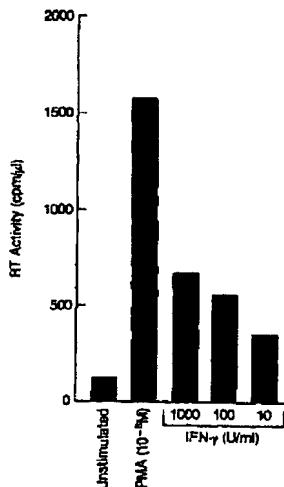
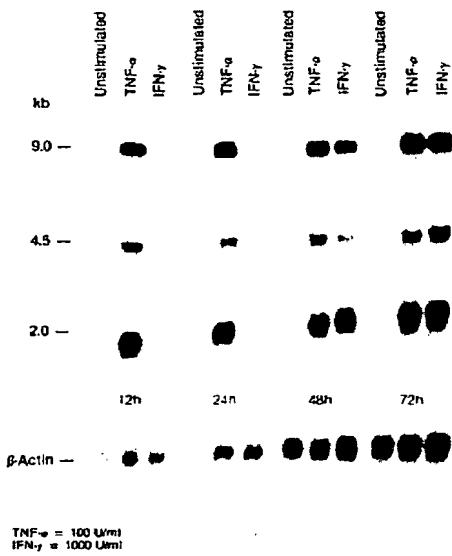


Figure 1. IFN- γ induces HIV expression in U1 cells. U1 cells were cultured in either unstimulated conditions or with various concentrations of IFN- γ . Culture supernatants were collected after 72 h stimulation and tested for the presence of RT activity. The results are the average of duplicate samples per each culture condition and are representative of at least 10 separate experiments. Similar results were obtained by determination of the levels of HIV p24 Ag in the culture supernatants, using a commercially available ELISA kit (Coulter Electronics, Hialeah, FL).

cumulation of all three species of HIV mRNA, including high levels of the unspliced 9-kb mRNA coding for gag-pol structural protein (Fig. 2), suggesting that IFN- γ may have a direct inductive effect on HIV transcription in monocytic cells, albeit with delayed kinetics as compared with TNF- α (Fig. 2) or PMA (data not shown).



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Figure 2. IFN- γ induces the accumulation of both spliced and unspliced HIV mRNAs. Total RNA was extracted from unstimulated U1 cells or from cells stimulated with either IFN- γ (1,000 U/ml) or TNF- α (100 U/ml) after 12, 24, 48, and 72 h of incubation, as described. IFN- γ induced significant levels of HIV RNA, although with delayed kinetics compared with TNF- α -stimulated cells.

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The Upregulatory Effect of IFN- γ on HIV Expression Is Not Mediated by the Production of Endogenous TNF- α or TNF- β . The observation that the induction of steady-state HIV mRNA by IFN- γ had delayed kinetics as compared with TNF- α or PMA suggested the possibility that the effect of IFN- γ could be mediated by the production of a second cellular factor. In this regard, we have previously reported that stimulation of U1 cells with PMA resulted in the synthesis and release of TNF- α , which actually mediated most of PMA-induced virus production (43). Therefore, we investigated the possibility that stimulation of U1 cells with IFN- γ , similar to PMA, triggered an autocrine pathway of viral induction mediated by endogenous TNF- α or TNF- β ; this possibility was also supported by the well-described ability of IFN- γ to induce TNF production in different cell types (reviewed in references 1–4), including MP (44). However, in contrast to cells stimulated with PMA, no evidence of TNF- α secretion (Fig. 3 A) and/or gene transcription (data not shown) were obtained in U1 cells stimulated with IFN- γ . Anti-TNF- α and anti-TNF- β Ab completely suppressed the induction of HIV expression mediated by their respective cytokine, but did not affect the ability of IFN- γ to induce HIV expression in U1 cells (Fig. 3 B). In addition, an anti-TNF- α mAb that significantly reduced the accumulation of HIV mRNAs in both TNF- α -stimulated and PMA-stimulated U1 cells did not affect IFN- γ induction of viral messages (data not shown).

Ultrastructural Studies of HIV Particle Synthesis in U1 Cells Stimulated with IFN- γ in the Absence and Presence of PMA. IFN- γ is a well-known activator of MP function (1–3). In addition, this lymphokine is known to exert profound effects on the differentiation of MP, including immature elements such as U937 promonocytic cells (the parental uninfected cell line of U1) (44–46). Because similar or even more profound effects on phagocyte activation and differentiation, as well as HIV expression, have been described after treatment with phorbol esters (46–48), we compared IFN- γ and PMA with regard to the influence of their cellular differentiative effects on the process of virion maturation in U1 cells. In this regard, we have previously described that PMA stimulation of U1 cells results in the rapid acquisition of the morphological (increased size, irregular plasma membrane, eccentric nucleus, prominent Golgi compartment, increased number of lysosomes) and functional (adherence to plastic surface, ability to secrete cytokines) characteristics of differentiated macrophages (38, 43). In addition, PMA stimulation of U1 cells induced budding and release of virions both at the plasma membrane as well as in Golgi-derived intracytoplasmic vacuoles, resembling effects independently described in primary human macrophages infected with HIV in vitro (28–30) or in vivo (31). IFN- γ stimulation of U1 cells resulted in distinct features of cell differentiation along the MP lineage similar to those observed after PMA treatment (Fig. 4, A and B). Furthermore, HIV particle production after IFN- γ stimulation was demonstrated both at the plasma membrane (Fig. 4, B and C) as well as in intracytoplasmic vacuoles (Fig. 4, B and D). However, these features were clearly observed only in a fraction (<10%) of U1 cells stimulated with IFN- γ . This

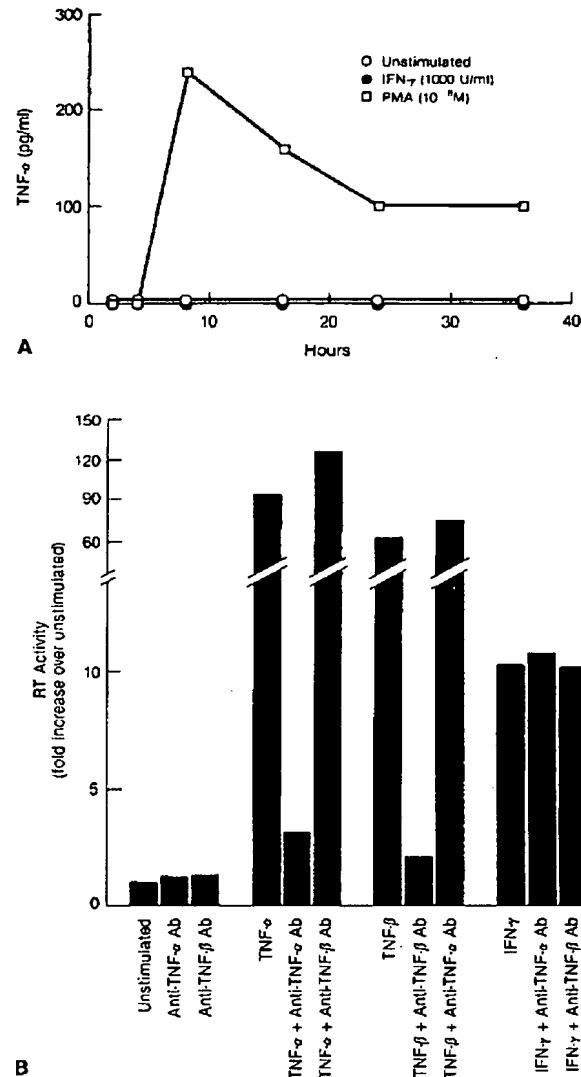


Figure 3. IFN- γ induction of HIV expression is not mediated by the secretion of endogenous TNF- α . (A) PMA stimulation of U1 cells induced the secretion of detectable levels of TNF- α , whereas no significant levels of this cytokine were observed in either unstimulated cells or cells stimulated with IFN- γ . Furthermore, IFN- γ did not inhibit PMA-induced secretion of TNF- α (not shown). The levels of TNF- α present in the culture supernatants were determined by the use of an ELISA kit (Quantikine, R & D Systems, Minneapolis, MN). (B) Effect of anti-TNF- α mAb (Cutter Biological, Miles Inc., Berkeley, CA) and anti-TNF- β -neutralizing Ab (R & D Systems) on HIV expression in U1 cells. Anti-TNF- α mAb (1 μ g/ml) and anti-TNF- β polyclonal Ab (50 μ g/ml) completely neutralized the inductive effect of TNF- α or TNF- β on HIV expression, respectively, without affecting IFN- γ -mediated upregulation of RT activity.

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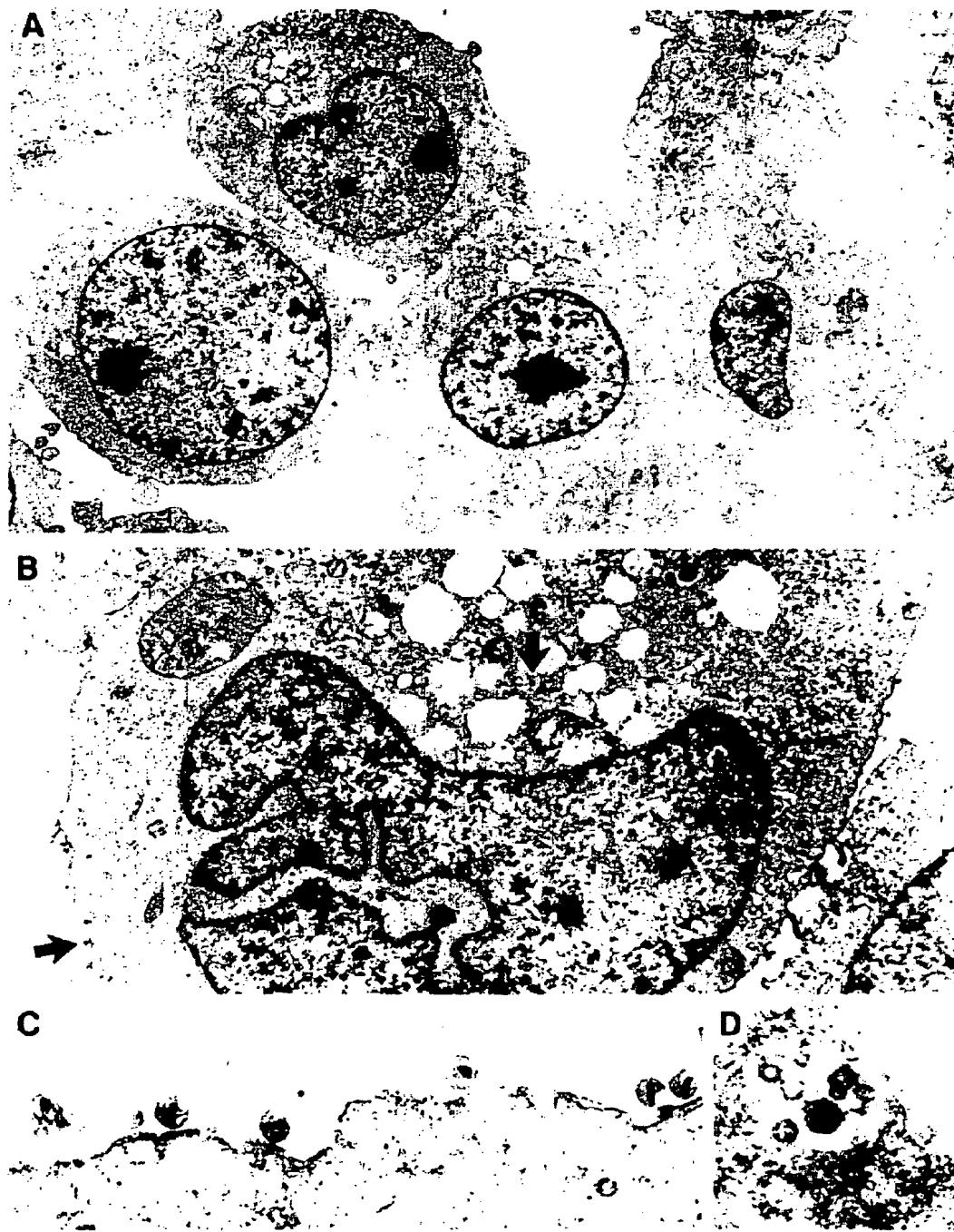
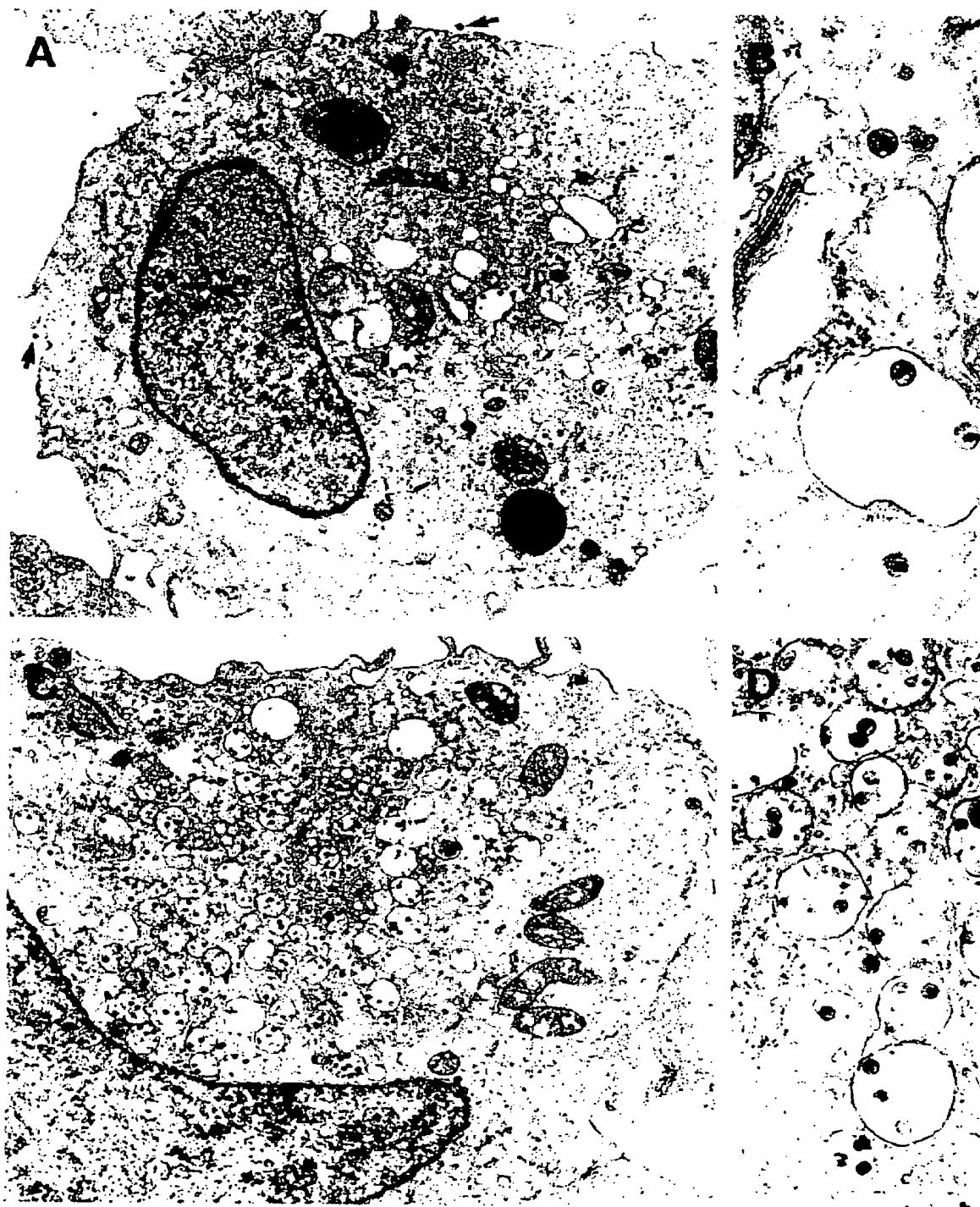


Figure 4. IFN- γ induces HIV particle expression and U1 cell differentiation. (A) Transmission electron microscopy of unstimulated U1 cells. The cytoplasm is dominated by ribosomes with only scattered mitochondria and profiles of rough endoplasmic reticulum and inconspicuous Golgi zones. (B) A portion of a U1 cell stimulated with IFN- γ for 72 h. Small clusters of virions are seen at several locations on the plasma membrane (arrow points to one cluster on bottom left, enlarged in C). The Golgi region is filled with vacuoles, one of which contains a mature virion (arrow, enlarged in D). (A) $\times 4,200$; (B) $\times 10,000$; (C) $\times 49,000$; (D) $\times 66,000$.

743 Biswas et al.

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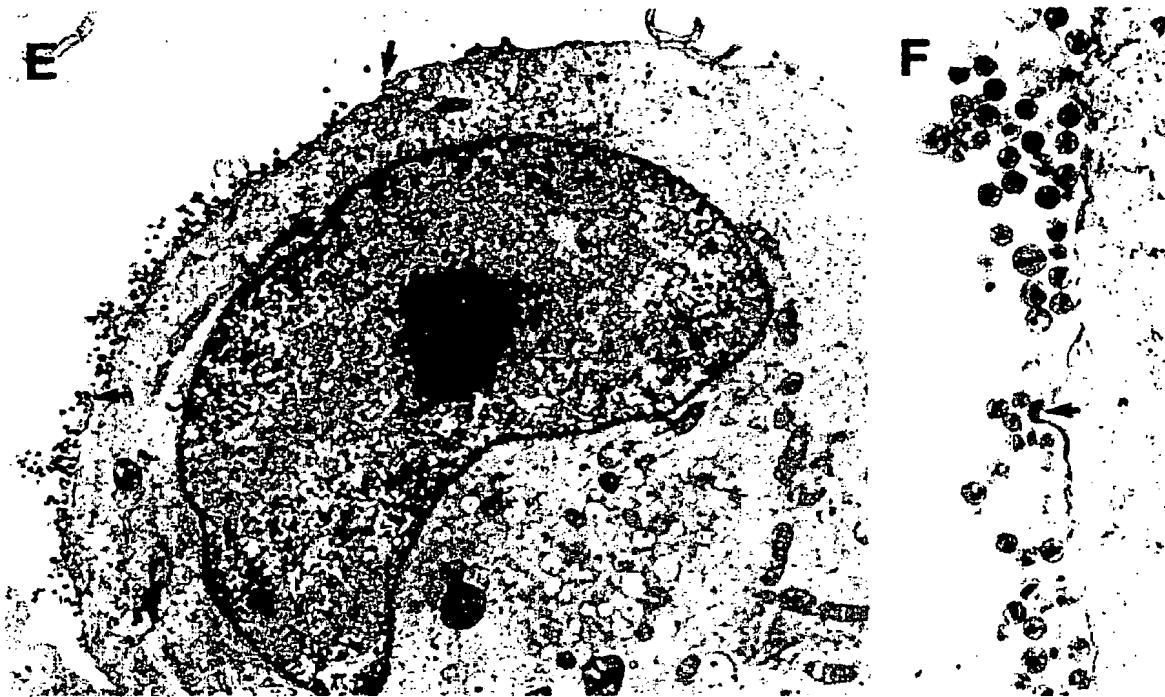
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744 IFN- γ Induces Expression of HIV in Mononuclear Phagocytes

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Figure 5. Effect of IFN- γ and IFN- α on PMA stimulated U1 cells. U1 cells were stimulated with PMA (10^{-8} M) either in the presence or absence of IFN- γ (1,000 U/ml) or IFN- α (100 U/ml) for 48 h. Cells were then fixed and analyzed by transmission electron microscopy. (A and B) PMA stimulation of U1 cells induced viral particle production both at the plasma membrane as well as in intracytoplasmic compartments. As compared with the unstimulated U1 cells (Fig. 4 A), PMA stimulation causes the cell to become more irregular in outline, with an eccentric indented nucleus, and a more prominent Golgi region. Small numbers of typical HIV particles are seen associated with the plasma membrane (arrows) and within Golgi-derived cytoplasmic vacuoles, some of which are enlarged in B. (C and D) IFN- γ decreased the levels of plasma membrane-associated virus, but consistently increased the intracellular accumulation of virions in cells stimulated with PMA. The Golgi region is gently expanded by vacuoles containing HIV (several enlarged in D). No HIV particles were detected on the surface of this cell in this section. (E and F) IFN- α caused both the accumulation of virions in intracellular vacuoles as well as their aggregation on the plasma membrane. A stretch of the plasma membrane is covered by multiple layers of typical HIV particles, at least two of which are in the budding stage (arrows). The Golgi region (lower right of E) is expanded by many vacuoles. (A) $\times 9,400$; (B) $\times 39,000$; (C) $\times 9,700$; (D) $\times 32,000$; (E) $\times 8,400$; (F) $\times 32,000$.

is different from stimulation of U1 with PMA in which similar morphological and functional changes were seen in the majority of the cells. We therefore investigated the ability of IFN- γ to modulate virus production in cells stimulated with PMA. In this regard, PMA and IFN- γ have been described as exerting synergistic effects on a variety of functions of MP cells, including the U937 cell line (45, 49). After 48 h of PMA stimulation, ~50–85% of U1 cells expressed typical HIV particles at the plasma membrane and/or in intracytoplasmic compartments (Fig. 5, A and B) compared with unstimulated cells in which virion production was essentially undetectable (Fig. 4 A). When U1 cells were stimulated with both PMA and IFN- γ , no changes in terms of the total number of cells expressing virus was observed. However, costimulation with PMA and IFN- γ compared with stimulation with PMA alone caused a threefold decrease in the number of cells expressing HIV exclusively at the plasma

membrane level, and a concomitant fourfold increase in cells in which particles were detected only in intracytoplasmic vacuoles (Figs. 5, C and D, and 6 A). This effect was demonstrated both in terms of a shift in the proportion of cells with intravacuolar virus as well as in the total number of virions present within the vacuoles (Fig. 5, C and D).

IFN- γ -mediated Accumulation of Virions in Intracytoplasmic Vacuoles Results in Decreased HIV Production in PMA-stimulated U1 Cells. Having established that costimulation of U1 cells with PMA and IFN- γ induced a redirection of the preferential site of virus expression from the plasma membrane to intracytoplasmic vacuoles, we next investigated the kinetics of this phenomenon. A progressive increase in the levels of vacuole-associated HIV particles was observed up to 5 d after U1 cell stimulation with either PMA or PMA plus IFN- γ (in which cultures this phenomenon was significantly more pronounced) (Fig. 6 B). In contrast, a reduction of plasma

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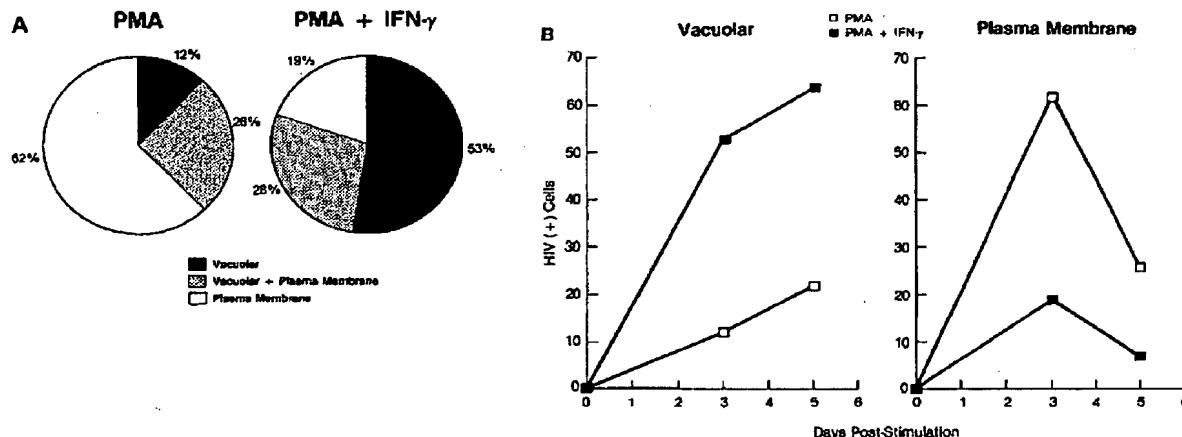


Figure 6. IFN- γ increases the intracellular accumulation of HIV particles in PMA-stimulated U1 cells. (A) A relative increase in the percentage of cells exclusively showing virions budding from and contained within intracytoplasmic vacuoles was observed in cells costimulated with PMA and IFN- γ compared with cells stimulated with PMA alone, as shown in Fig. 5. Results were quantified by transmission electron microscopy after 72 h of stimulation. The results are representative of three independent experiments. (B) Kinetics of virion accumulation at the plasma membrane and in intracytoplasmic vacuoles. The results are obtained by the analysis of the morphological features of 100 consecutive cells from one representative experiment out of three independently conducted. The simultaneous expression of virions in vacuoles and at the plasma membrane (not shown) was relatively stable in that it was seen in \sim 30% of U1 cells stimulated with either PMA alone or with PMA plus IFN- γ both after 3 and 5 d of culture.

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membrane associated virus was seen in cells stimulated with PMA in the presence of IFN- γ as compared with cells induced by PMA alone (Fig. 6B). Furthermore, from a kinetic standpoint, virus tended to accumulate over time in the vacuolar compartment as opposed to the plasma membrane whether cells were stimulated with PMA alone or with PMA plus IFN- γ . This was underscored by the observation that lower levels of HIV were exclusively associated with the plasma membrane after 5 d compared with 3 d of culture regardless of the stimuli, while the levels of virions segregated exclusively in vacuoles increased from day 3 to day 5 in culture (Fig. 6B). We further investigated whether these morphological changes had a functional impact on the ultimate release of HIV from the infected cells. In this regard, we have previously confirmed that measurement of RT activity production from U1 cells (as well as other cell lines persistently infected with HIV) is a valid parameter of HIV particle release, in that no detectable RT activity was demonstrable in column fractions containing particle-free viral proteins (such as p24 Ag) shed from infected cells (50). A significant decrease of the RT activity levels released by U1 cells stimulated with PMA in the presence of IFN- γ was observed compared with cells stimulated with PMA alone (Fig. 7), suggesting that the IFN- γ -mediated redirection of virus production within intracytoplasmic vacuoles resulted in a relatively stable intracellular sequestration of virions throughout the culture period.

IFN- γ Does Not Suppress the Synthesis of HIV mRNAs and Proteins in PMA-stimulated Cells. Although the ultrastructural analysis of U1 cells stimulated with PMA in the presence of IFN- γ did not indicate that this lymphokine had a suppressive effect on HIV biosynthesis, this technique allows one to examine only a small fraction of the total cell popula-

tion. We therefore investigated whether the reduced levels of RT activity observed in PMA-stimulated cells in the presence of IFN- γ could have been caused by reduced HIV mRNA and/or protein synthesis. No significant differences were observed between the levels of HIV mRNA in U1 cells stimu-

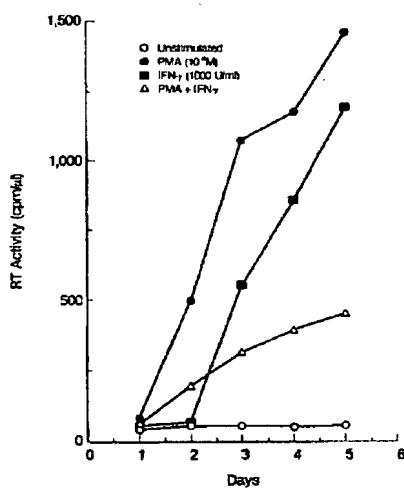


Figure 7. Suppressive effect of IFN- γ on PMA induction of RT activity in U1 cell. Kinetic analysis reveals that although IFN- γ alone induces RT activity in U1 cells, the stimulation with PMA and IFN- γ markedly suppressed the induction of RT activity compared with stimulation with PMA alone.

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lated with PMA or PMA plus IFN- γ , as shown by quantitative slot-blot analysis (Fig. 8A). Furthermore, no significant changes in the relative or absolute levels of spliced and unspliced HIV mRNAs were seen by Northern blot analysis of these cells (data not shown). A modest increase in the levels of cell-associated HIV proteins was observed in U1 cells stimulated with PMA plus IFN- γ as compared with cells activated with PMA alone, likely resulting from the independent inductive effect of these two agents (Fig. 8B), and/or from the increased accumulation of vacuole-associated HIV particles in cells costimulated with PMA and IFN- γ (Figs. 5 and 6). Finally, cell disruption by multiple cycles of freezing and thawing resulted in the recovery of RT levels from U1 cells costimulated with PMA and IFN- γ to the levels of cells stimulated with PMA alone (data not shown), indicating that the reduced levels of supernatant-associated RT activity were not a consequence of decreased virus expression, but resulted from the accumulation of particles in intracellular compartments.

The Decrease in PMA-induced Virus Production in U1 Cells Stimulated with IFN- γ Is Not Mediated by Secretion of Endogenous IFN- α/β . We and others have previously described that IFN- α could suppress the production of RT activity in persistently infected T lymphocytic cell lines as a consequence of a block in the release of HIV particles from the plasma membrane and in the absence of a suppressive effect on HIV protein synthesis (33–35). To investigate whether the reduced production of HIV particles in the culture supernatant observed in cells stimulated with PMA in the presence of IFN- γ was a consequence of endogenously produced IFN- α , we compared the effect of IFN- α with that of IFN- γ in U1 cells stimulated with PMA at the ultrastructural level. Similar to IFN- γ , IFN- α increased the intracellular accumulation of virus particles in PMA-stimulated U1 cells (Fig. 5, E and F). How-

ever, unlike IFN- γ , which decreased the quantity of plasma membrane-associated virus, IFN- α caused the clustering of virions at the external plasma membrane (postbudding effect), as previously described in several infected T lymphocytic cell lines (33–35). Both IFN- α and IFN- γ inhibited PMA-induced virus expression, although IFN- α was consistently more potent in this regard than IFN- γ (Fig. 9). Anti-IFN- α Ab completely restored the levels of RT activity in the culture supernatants of cells stimulated with PMA plus IFN- α to the levels observed in cells stimulated with PMA alone, whereas it did not affect the suppressive effect of IFN- γ (Fig. 9). Similar results were observed in U1 cells incubated under these conditions for longer times, and in experiments using rIFN- β and anti-IFN- β -neutralizing Ab (data not shown), indicating that the suppressive effects of IFN- γ on RT production in PMA-stimulated U1 cells were not mediated by IFN- α or IFN- β . Of note is the fact that no inductive effects on HIV expression were seen in U1 cells treated with IFN- α alone (or IFN- β alone; data not shown), in contrast to cells stimulated with IFN- γ .

Discussion

In the present study we have demonstrated that IFN- γ is a direct inducer of HIV expression in persistently infected promonocytic U1 cells. The effect of IFN- γ was correlated

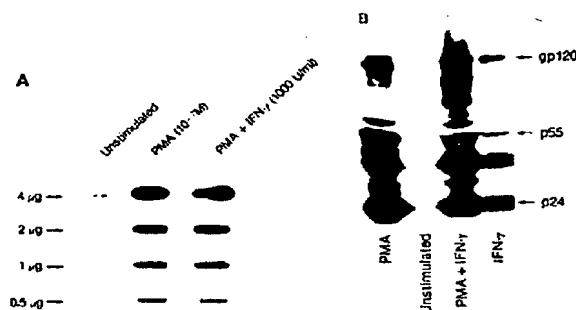


Figure 8. IFN- γ does not reduce the levels of PMA-induced HIV mRNA or proteins. (A) Slot-blot analysis of HIV RNA. Total RNA was extracted from either unstimulated U1 cells or from cells stimulated with PMA or PMA plus IFN- γ for 24 h, and diluted in a slot-blot apparatus. Northern blot analysis confirmed that IFN- γ did not reduce PMA-induced accumulation of spliced and unspliced HIV mRNAs. (B) Direct induction of HIV protein synthesis in U1 cells stimulated with IFN- γ alone and lack of IFN- γ inhibition of PMA-induced viral proteins. The results represent the levels of cell-associated HIV proteins present after 48 h incubation and were confirmed by analyses performed at both earlier (24 h) and later (72 h) times after stimulation.

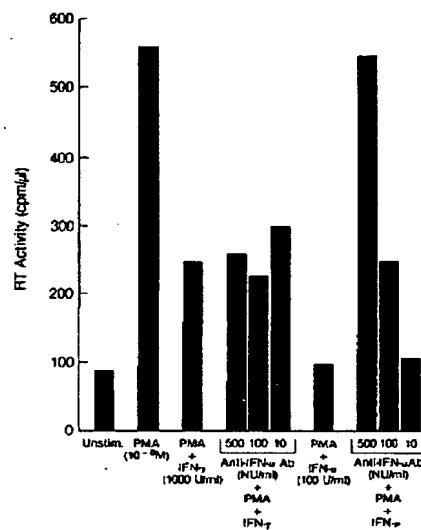


Figure 9. IFN- γ inhibition of PMA-induced HIV expression is not mediated by the release of endogenous IFN- α . U1 cells were stimulated with PMA in the presence or absence of IFN- α or IFN- γ , either alone or in combination with various concentrations of a sheep anti-IFN- α polyclonal Ab (Interferon Sciences, Inc., New Brunswick, NJ). The results refer to the levels of RT activity detected after 48 h of incubation, when the inductive effect of IFN- γ alone was not yet detectable. No effect of the anti-IFN- α Ab was seen on cells treated with PMA plus IFN- γ even at later time points.

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with the accumulation of spliced and unspliced HIV mRNAs as well as with viral protein synthesis. In addition, IFN- γ induced the differentiation of U1 cells along the MP lineage in association with features of intracytoplasmic HIV accumulation in Golgi-derived vacuoles. Furthermore, IFN- γ greatly enhanced the intracellular accumulation of HIV concomitant with a significant reduction of plasma membrane-associated virions in U1 cells costimulated with PMA, a potent inducer of both HIV transcription and macrophage differentiation.

Several cytokines have been described as being capable of either upregulating (i.e., IL-2, IL-3, IL-6, M- and GM-CSF, and TNF- α/β), suppressing (i.e., IFN- α/β), or exerting multiple and opposite effects (TGF- β and IL-4) on HIV expression (reviewed in references 21 and 37). With regard to IFN- γ , Koyanagi et al. (20) observed that stimulation of MDM with this lymphokine before infection induced viral replication, whereas IFN- γ treatment of previously infected MDM reduced HIV expression; however, no explanation for this functional dichotomy was provided, and different results were reported by other investigators (19). In the persistently infected U1 cells employed in the present study, we have investigated by both functional and ultrastructural studies the patterns of virus production after stimulation with IFN- γ alone or after costimulation with IFN- γ and PMA. The direct inductive effect of IFN- γ on HIV expression was correlated with cell differentiation and the appearance of virions in intracellular vacuoles, although these features were clearly present only in a minority of the cell population. In PMA-stimulated cells, IFN- γ caused a significant inhibition of virion-associated RT activity released into the culture supernatant as compared with cells stimulated with PMA alone. However, this effect was not explained by either a reduced synthesis of viral proteins or RNA. When the functional and morphological effects of IFN- γ were compared with those of IFN- α , we observed that both IFNs modulated virus production in persistently infected cells stimulated with PMA by either inhibiting the ability of the virus to be released from the plasma membrane (as previously seen in IFN- α -treated T lymphocytic cells and here demonstrated in monocytic U1 cells) or by causing an accumulation of virions in intracellular vacuoles (as here shown in U1 cells treated with either IFN- α or IFN- γ). These results suggest that the capability of IFN- γ to shift the preferential morphological site of virus maturation from the plasma membrane to the intracellular compartment leads to an apparent decrease in particle release, which in reality is a diversion of

virion production resulting in a relatively stable intracellular viral reservoir. In this regard, we have previously described that long-term *in vitro* cultures of bone marrow-derived human macrophages infected *in vitro* with HIV-1 contained abundant levels of vacuole-associated particles after several weeks of culture (51). Furthermore, other investigators have observed virus accumulation in intracellular compartments as well as liberation of substantial RT activity after cell disruption after several days of culture of primary infected MDM (28–30, 32). Finally, vacuole-associated HIV has also been described in the brain macrophages of HIV-infected individuals (31). The potential role of IFN- γ as an *in vivo* mediator of intracellular accumulation of virus particles in monocytes and macrophages of HIV-infected individuals remains to be established. However, the observation of elevated levels of IFN- γ (or of its related marker neopterin) in the plasma and CSF (5–7) as well as in the lymph nodes of HIV-infected individuals (12), where the number of infected cells actively expressing HIV is much higher compared with the peripheral blood compartment (52–54), suggests that IFN- γ may play a role in the *in vivo* regulation of HIV expression.

Although IFN- γ and TNF- α have now been clearly demonstrated to induce HIV expression in a variety of model systems, including IL-2-stimulated PBMC (14), earlier studies had indicated the possibility that these two cytokines, either alone or in combination, could exert some suppressive effect on virus replication (15). In addition, IFN- γ restored certain defective functions *in vitro*, such as the natural killer cell activity in PBMC of HIV-infected individuals (10, 55). Based on these and other observations (15–19), IFN- γ has been employed as a therapeutic agent both in the treatment of HIV infection and of AIDS-associated Kaposi's sarcoma (reviewed in references 56 and 57) with conflicting results. In some studies reduction of plasma levels of p24 Ag, improvement of immune function, and clinical course have been described (58), whereas exacerbation and progression of disease have also been reported (59). Our *in vitro* findings suggest that the inductive effects of IFN- γ on HIV-infected MP are complex and that the apparent suppressive effects on virus production may in fact represent merely a diversion of the preferential subcellular site of virion production from the plasma membrane to intracytoplasmic vacuoles. In addition, we have clearly demonstrated that IFN- γ alone can upregulate HIV expression in persistently infected MP cells. Thus, caution should be used in the design of therapeutic strategies involving IFN- γ as potential antiviral agent.

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Cytokines alter production of HIV-1 from primary mononuclear phagocytes.

Koyanagi Y, O'Brien WA, Zhao JQ, Golde DW, Gasson JC, Chen IS.

Department of Microbiology and Immunology, UCLA School of Medicine 90024.

Some strains of human immunodeficiency virus type 1 (HIV-1) can infect primary monocytes and monocyte-derived macrophages in vitro. In this report, the effect of cytokines on the production of one of these strains that shows a tropism for mononuclear phagocytes, designated HIV-1JR-FL, was studied. Primary peripheral blood mononuclear phagocytes infected with HIV-1JR-FL were treated with the hematopoietic factors: granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), macrophage colony-stimulating factor (M-CSF), and gamma-interferon (gamma-IFN). The M-CSF, GM-CSF, IL-3, and gamma-IFN were able to alter HIV-1 production under different conditions.

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**INTERFERONS AND BACTERIAL LIPOPOLYSACCHARIDE
PROTECT MACROPHAGES FROM PRODUCTIVE INFECTION
BY HUMAN IMMUNODEFICIENCY VIRUS IN VITRO**

By RICHARD S. KORNBLUTH, PHILIP S. OH, JAMES R. MUNIS,
PATRICK H. CLEVELAND, AND DOUGLAS D. RICHMAN

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San Diego, and the Veterans Administration Medical Center, San Diego, California, 92161*

Macrophages are now recognized to be important cellular targets in HIV infection. These cells can be infected with HIV *in vitro* (1-7) and express HIV antigens and nucleic acid *in vivo* (8-13). Macrophage-tropic forms of HIV can be isolated from brain, lung, and blood (4, 7, 14), and these isolates replicate for months in monocyte-derived macrophages *in vitro* (4). Unlike CD4 T cells, macrophages are not rapidly killed by HIV and may serve as a persistent viral reservoir. HIV-infected macrophages may also have a critical role in the immunological disturbances and encephalopathy of HIV infection (15-17). Furthermore, cultured macrophages contain reduced levels of the kinases required to activate dideoxynucleosides such as AZT, and may not be protected by these agents as efficiently as T cells (18). Thus, additional therapeutic strategies for HIV infection may be needed in order to address the special biology of HIV infection in macrophages.

Numerous studies have demonstrated the effect of various stimuli upon macrophage functions (19, 20). Endotoxin, in the form of bacterial lipopolysaccharide (LPS), profoundly affects many macrophage functions (21). Also, endogenous cytokines such as interferons may physiologically "activate" macrophages. It has been proposed that the depletion of IFN- γ -producing T cells in AIDS leads to a defect in macrophage activation (22), and consequently IFN- γ is now entering clinical trials in AIDS patients. IFN- α and IFN- β are already being tested in patients. However, the possibility that these interferons have antiviral effects against HIV in macrophages has not been examined. In this study, we demonstrate that treatment with IFN- α , IFN- β , IFN- γ , or LPS prevents the establishment of productive HIV infection in cultured macrophages.

Materials and Methods

Macrophage Culture. PBMC were isolated from the blood of healthy volunteers by centrifugation over Ficoll-Hypaque. Monocytes were initially isolated by adherence to fibronectin (23). These cells were 70-90% nonspecific esterase (NSE)¹-positive (Kit 90-A1; Sigma Chemical Co.).

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¹ Abbreviations used in this paper: GM-CSF, granulocyte/macrophage colony-stimulating factor; M-CSF, macrophage CSF; MDM, monocyte-derived macrophages; MOI, multiplicity of infection; NSE, nonspecific esterase; TCID₅₀, 50% tissue-culture infectious dose.

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1137

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1138 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES

ical Co., St. Louis, MO) and were further purified by adherence to plastic. Initially, 4×10^5 fibronectin-adherent cells were cultured in 1 ml of RPMI 1640 (Whittaker M. A. Bioproducts, Inc., Walkersville, MD or Irvine Scientific, Santa Ana, CA) containing 10% heat-inactivated autologous or AB human serum and 50 μ g/ml gentamicin in the 1-cm² wells of 48-well plates. All culture materials were endotoxin-free in two assays (the *Limulus* lysate assay and a sensitive monocyte procoagulant activity assay) as previously described (22). Fibronectin-adherent cells, enriched in monocytes, were cultured at 37°C in 5% CO₂/95% air for 5–9 d to allow them to mature into fully adherent cells (4). An additional purification step occurred when the monolayers were washed four times at the time of infection (see below). The final adherent cells ($\sim 2 \times 10^5$ /well) were 100% NSE⁺ and are referred to as "monocyte-derived macrophages" (MDM).

HIV Infection. The HTLV-III_{Ba-L85} macrophage-tropic isolate of HIV-1 was the gift of Drs. S. Gartner and M. Popovic, National Cancer Institute, Bethesda, MD (4). The third passage of this virus in MDM under endotoxin-free conditions in our laboratory was used to make a virus pool which was aliquoted and stored at -70°C. This pool had an infectivity titer by terminal dilution assay in MDM (as determined by p24 core antigen production) of 10^6 50% tissue-culture infectious doses (TCID₅₀)/ml. This isolate fails to replicate in T cell lines (CEM and MT-2) that support many T lymphotropic strains of HIV-1.

MDM were infected with HIV by replacing their medium with 0.2 ml per well of HIV in medium containing 10% FCS (HyClone Laboratories, Inc., Logan, UT) instead of human serum. The multiplicity of infection (MOI) ranged between 0.1 and 1 in different experiments as indicated. After at least 2 h of incubation to permit viral adsorption, unadsorbed virus and residual nonadherent cells were removed by washing each well four times with 1 ml medium. Wells were refed 1 ml medium containing FCS and the indicated treatment, and incubated for the times shown.

Interferons, Cytokines, and LPS. Additions to culture medium were made before infection with HIV, during infection, or after infection, as indicated. rIFN- α (also known as IFN- α 2a, IFN- α 2, or Roferon) (2×10^8 U/mg) was obtained from Hoffmann-La Roche, Nutley, NJ. rIFN- β (1.8×10^8 U/mg) was the gift of Dr. C. Budd Colby, Triton Biosciences, Inc., Alameda, CA. rIFN- γ (1.6×10^7 U/mg) was purchased from Amgen, Thousand Oaks, CA. rIL-1 β and IL-4 were obtained from Genzyme Corp., Boston, MA. rIL-2 (7.3×10^5 U/mg) was obtained from New England Nuclear, Boston, MA. Recombinant macrophage colony-stimulating factor (24) (M-CSF) was the gift of Dr. P. Ralph, Cetus Corp., Emeryville, CA. Recombinant granulocyte/macrophage CSF (GM-CSF) (6.7×10^6 U/mg), M-CSF (25) (8×10^5 U/mg), and IL-6 (5×10^6 U/mg) were gifts from Dr. Steven C. Clark, Genetics Institute, Cambridge, MA. (GM-CSF was also obtained from Genzyme Corp. and used in preliminary experiments). rTNF- α (3.02×10^7 U/mg) and lymphotoxin (TNF- β) (1.28×10^6 U/mg) were gifts from Dr. H. Michael Shepard, Genentech, Inc., South San Francisco, CA. Phenol-extracted LPS from *Escherichia coli* 0111:B4 was from Calbiochem-Behring Corp., La Jolla, CA.

Crude "macrophage-activating factor" was prepared from PBMC by stimulation with Con A-Sepharose (26). After 48 h, the supernatant medium was centrifuged and stored at 4°C for no more than a week. Control supernatant was prepared in the same manner except that Con A-Sepharose was omitted.

Viral Production and Cytopathology Assays. Cells were incubated for the times indicated and 0.05–0.1-ml aliquots were removed without refeeding and stored at -20°C for later assay of p24 core antigen by ELISA (Abbott Laboratories, N. Chicago, IL). Additional aliquots were stored at -70°C and subsequently assayed for infectious HIV by terminal dilution assay, as described above. Wells were also serially observed by phase-contrast microscopy and scored for the presence of multinucleated giant cells (≥ 4 nuclei per cell).

Electron Microscopy. Macrophage cultures in Costar (Cambridge, MA) six-well plates were fixed for 24 h in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate, washed for 1 h in phosphate buffer, and fixed for 30 min with 2% osmium tetroxide (buffered as for the glutaraldehyde). The fixed wells were then dehydrated through a graded series of ethanol to 90% and then passed through a graded series of hydroxypropyl methacrylate from 90% in water to 100%. Next, wells were infiltrated with sequential mixtures of hydroxypropyl

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KORNBLUTH ET AL.

1139

methacrylate and Epon 812, starting at three parts to one part respectively and terminating in pure Epon 812 as previously described (27). Embedding was accomplished by quickly inverting and setting a Beem capsule filled with Epon 812 over areas of the culture that contained multinucleated giant cells or normal appearing areas. After polymerization for 48 h at 60°C, the wells were frozen at -70°C and the Beem capsules were snapped to remove the cells from the plastic dishes. The embedded cultures were mounted for sectioning either parallel or perpendicular to the culture surface. Initially, blocks were thick sectioned, stained with toluidine blue, and examined by light microscopy. Areas of interest were thin sectioned with a diamond knife and stained with saturated uranyl acetate in 50% ethanol followed by bismuth oxynitrate hydrate (28). The sections were examined on a Zeiss 10A electron microscope.

Nucleic Acid Hybridization Studies. Total cellular RNA was isolated from macrophage cultures using a modification of the single-step acid guanidinium thiocyanate-phenol-chloroform procedure (29) (RNAzol; CInna/Biotecx Laboratories International, Inc., Friendswood, TX). DNA was also obtained from this extraction and further purified using proteinase K and DNase-free RNase. RNA or DNA were blotted onto nitrocellulose, hybridized with a ³²P-labeled HIV-1 probe, pARV-2 (30), partial proviral clone of HIV-1sr2 (the gift of Dr. Paul Luciw, University of California, Davis, CA), and analyzed by autoradiography.

Results

Time Course of HIV Infection in Untreated Macrophages. In the absence of any treatment, MDM readily supported the replication of the HTLV-III_{La-L85} macrophage-tropic strain of HIV-1. Immediately after infection, supernatants routinely contained <30 pg/ml of p24 core antigen (the threshold of detection in this assay), indicating the effectiveness of the washing procedure used to remove free input virus (Fig. 1). By day 1, however, a small amount of p24 (100–300 pg/ml) appeared in all supernatants, and may represent residual virus that was inaccessible to the washing procedure. Thereafter, p24 antigen in the supernatant markedly increased by day 6 and reached a plateau level in ~10 d. The final levels of p24 antigen achieved varied between experiments by an order of magnitude, which may reflect differences between macrophage donors or the day of culture on which the cells were infected (data not shown).

Ultrastructure of HIV-infected Macrophages. HIV infection of untreated MDM led to the formation of multinucleated giant cells by day 6 (Fig. 2). When fixed and embedded on the culture dish and sectioned perpendicular to its surface, a polarization of virion assembly was seen. Virions were most abundant in a region characterized by intracytoplasmic vesicles containing numerous interdigitating microvilli,

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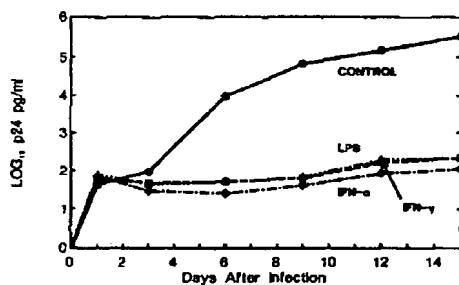
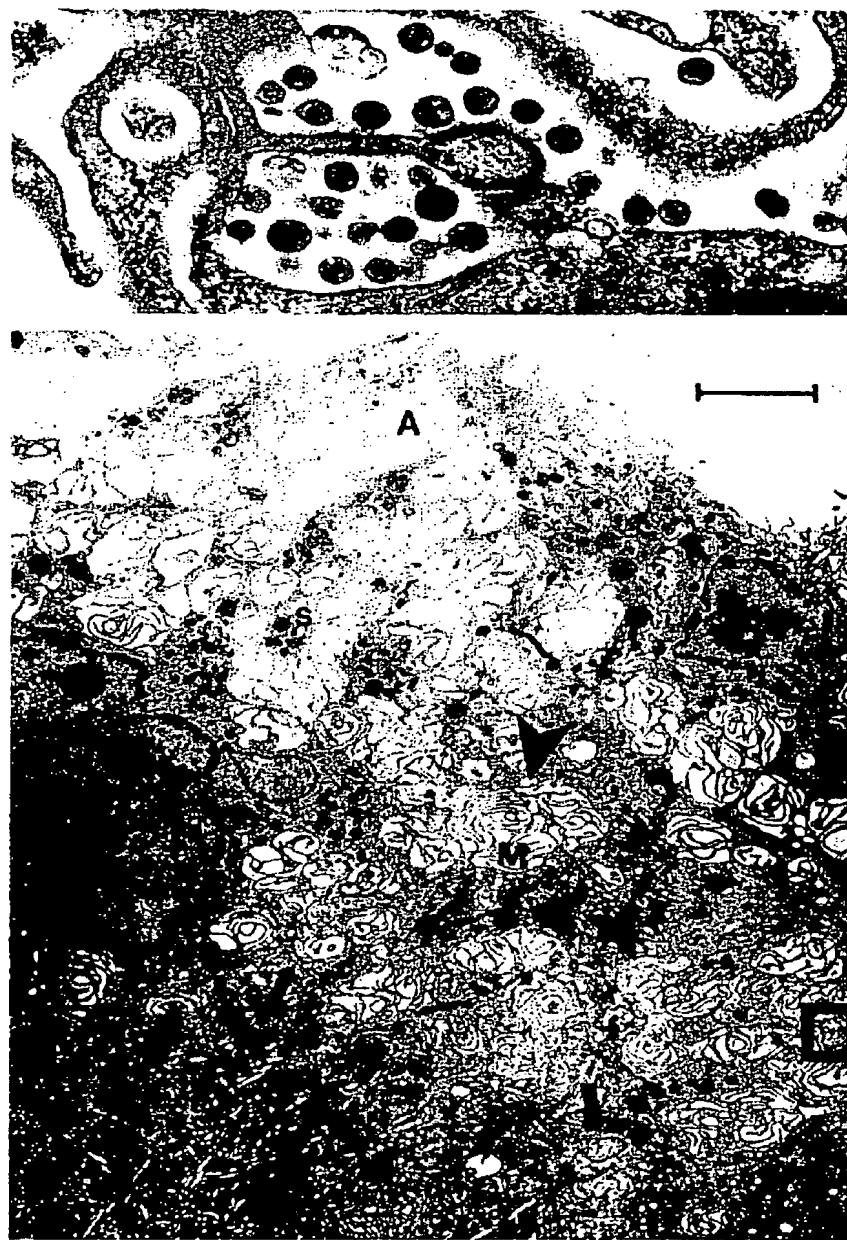


FIGURE 1. Semi-log plot of the time course of p24 core antigen accumulation in treated vs. control cultures. MDM were infected with HIV (MOI = 1), washed to remove input virus, and treated with IFN- α (5,000 U/ml), IFN- γ (5,000 U/ml), or LPS (10 μ g/ml). Aliquots of the supernatant medium were harvested at the times indicated after infection, and analyzed for p24 core antigen. The log₁₀ of the mean p24 pg/ml of duplicate cultures is shown.

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1140 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES



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KORNBLUTH ET AL.

1141

reminiscent of the plasmalemma found at the surface of macrophages. These areas appeared immediately apical to a region of very densely stained cytoplasm and extensive, dilated smooth endoplasmic reticulum $\sim 5 \mu\text{m}$ above the surface of the culture dish (Fig. 2). Proceeding apically toward the free surface of the giant cell, the intracytoplasmic vesicles became larger and the number of virions per vesicle decreased. Although virions were occasionally found budding from the plasma membrane, most budding particles were seen in small intracytoplasmic vesicles (Fig. 3, A-C). Characteristically, vesicles with the highest number of virions had an electron-dense coating (Figs. 2 and 3).

Cumulatively, more than a thousand virions were seen in sections from untreated, HIV-infected cultures. In contrast, no multinucleated giant cells or virions were seen in cultures treated with 1,000 U/ml of either IFN- α or IFN- γ , 100 ng/ml LPS, or in uninfected cultures (see below). Also, there was no evidence of incomplete virion assembly or virus accumulation inside the cells treated with interferon or LPS.

Effects of Interferons, LPS, and Cytokines on HIV Infection in Macrophages. To evaluate the effects of INF- α , IFN- γ , and LPS, these agents were added to macrophage cultures immediately after HIV infection. A single addition of each of these agents was capable of reducing p24 antigen accumulation by 1,000-fold (Fig. 1). Antigen accumulation was prevented and not merely delayed, which indicates that the production of additional virions was prevented. However, a low-level (<1,200 pg/ml) of p24 antigen was found in the media of treated cultures in all experiments. While this is compatible with the persistence of input virus not removed by the washing procedure, the possibility that some virus replication resists these treatments cannot be excluded.

A dose-response study was performed to quantitate these effects further (Fig. 4). In four experiments, treatment with 100–1,000 U/ml (30–300 pM) of IFN- α or 10–100 U/ml (25–250 pM) of IFN- γ 48 h before infection was sufficient to reduce viral production maximally, as measured by the accumulation of p24 antigen in the supernatant 14 d later. The slope of the dose-response to LPS was consistently less steep than that of the interferons, and 100 ng/ml was required to reduce p24 antigen release 10-fold. In contrast, IL-4 was much less effective, and the apparent effects at high doses could be attributed to endotoxin contamination (data not shown).

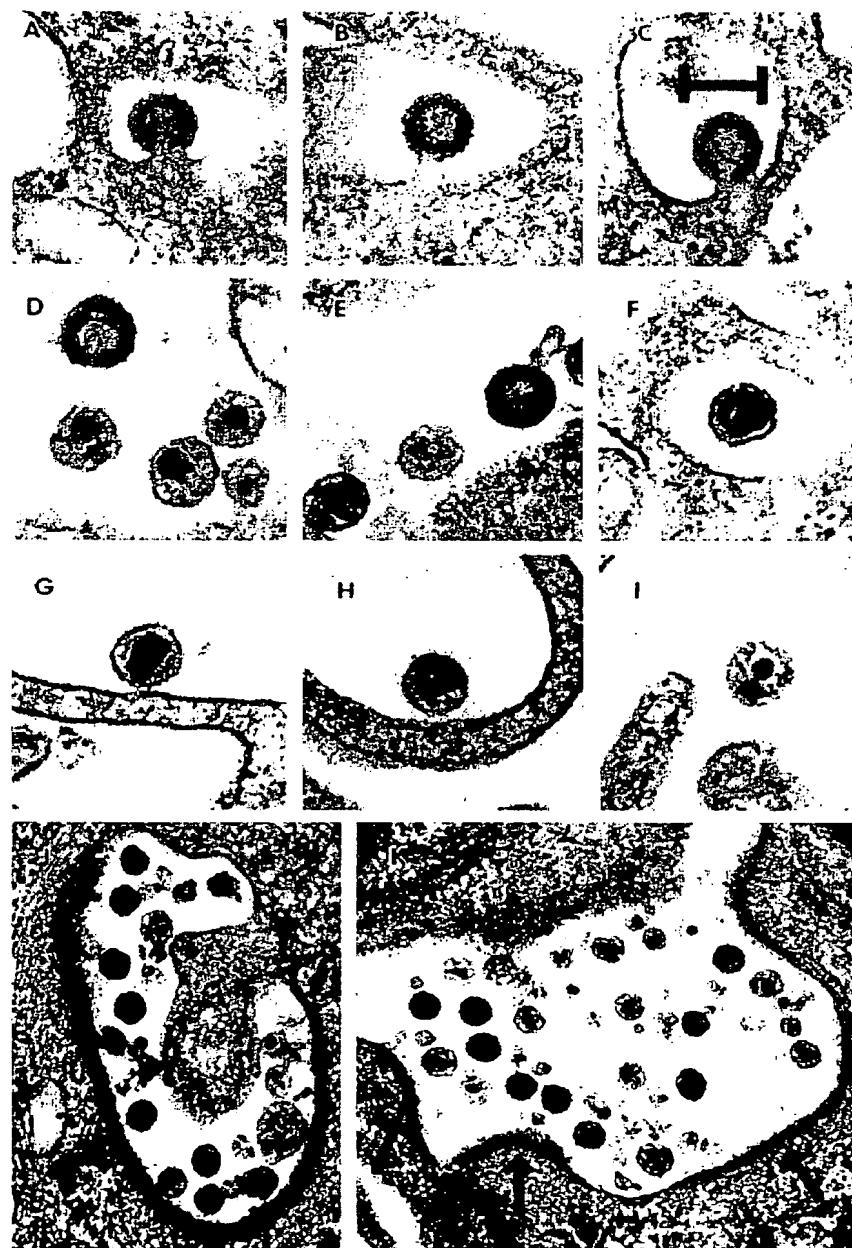
In separate experiments (Fig. 5), IFN- β at 10–100 U/ml (3–30 pM) also protected MDM from HIV. A single addition of M-CSF (from Cetus Corp.) (Fig. 5) or repeated

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FIGURE 2. Ultrastructure of HIV-induced multinucleated giant cells. MDM were infected with HIV at MOI = 1. 6 d later, the cultures were fixed and processed for transmission electron microscopy. In this figure, a single multinucleated giant cell from an untreated, infected culture was sectioned perpendicular to the culture dish surface to reveal the differences between basal (B), medial (M), and apical (A) areas of the cell. Examination of thick sections revealed at least 18 nuclei in this single cell (not shown). The basal portion of the cell was characterized by a dense cytoplasm and dilated smooth endoplasmic reticulum. The prominent intracytoplasmic vesicles were characterized by extensive interdigititation of microvilli (arrowheads) in the medial area of the giant cell. Progressing toward the apical (free) surface, the number of virions within these vesicles decreased and the amount of luminal space increased. Numerous intracytoplasmic vesicles with membranes containing electron-dense deposits (arrows) were also seen. $\times 3,200$; bar = $5 \mu\text{m}$. (Top) Enlargement of boxed area below. Numerous virions are seen within intracytoplasmic vesicles present in the medial layer of the cell. $\times 48,000$.

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1142 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES



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KORNBLUTH ET AL.

1143

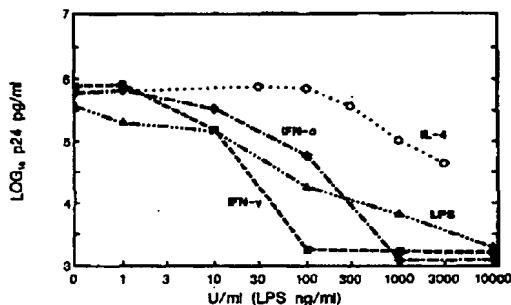


FIGURE 4 Log-log plot of the dose-response curves of anti-HIV treatments. MDM were pretreated for 48 h with the indicated doses of IFN- γ , IFN- α , LPS, or IL-4, then infected with HIV (MOI = 1), washed, and the treatments readded. Supernatants were collected for p24 core antigen assay 14 d later, and the \log_{10} of the mean p24 pg/ml of duplicate cultures is shown. Multinucleated giant cells were absent in cultures treated with $>1,000$ U/ml of IFN- α , >100 U/ml of IFN- γ , $>1,000$ ng/ml of LPS, and $>1,000$ U/ml of IL-4.

additions of M-CSF (data not shown) minimally enhanced the replication of HIV. (The larger form of M-CSF from Genetics Institute also did not affect HIV replication). However, because this study used an already highly permissive in vitro infection system, the replication-enhancing effects of cytokines such as M-CSF (6) may be masked by the high background. GM-CSF partially protected MDM from HIV in two experiments (one of which is shown in Fig. 5, using GM-CSF from Genetics Institute), but had no effect in two other experiments, and actually enhanced HIV replication in one experiment (data not shown). IL-1 β , IL-2, IL-6, TNF- α , and lymphotoxin (TNF- β) were inactive (Fig. 5 and Table I). In addition, unstimulated PBMC do not constitutively release into their supernatant any factor active in this experimental system, whereas the lymphokine-rich supernatant of Con A-stimulated PBMC (which contained IFN- γ ; data not shown) reduced p24 accumulation \sim 30-fold (Table I).

Since p24 core antigen accumulation might not adequately reflect the release of infectious virus, supernatants were used to inoculate additional macrophage indicator cultures, which in turn were evaluated for multinucleated giant cells and production of p24 antigen. Whereas supernatants from untreated cultures contained $>10^3$ TCID₅₀/ml, no infectious virus could be detected in the supernatants of cultures pretreated with IFN- γ (1,000 U/ml), IFN- α (1,000 U/ml), or LPS (1 μ g/ml). To show that this was not due to carry over of interferon or LPS into the indicator cultures, neutralizing antibodies or polymyxin B were added respectively, but still no infectious virus could be detected in the treated cultures.

Kinetics of Interferon and LPS Effects. To evaluate the stage of infection at which IFN- α , IFN- γ , or LPS acted, MDM were washed four times and treated with high doses of IFN- γ , or IFN- α or LPS initiated at increasing intervals after infection. Interferons or LPS added as late as 3 d after infection with HIV still decreased the release of p24 antigen into the supernatant (Fig. 6). By day 6 after infection, how-

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FIGURE 3. Ultrastructure of HIV budding. Montage from the same culture as Fig. 2. Virions were seen budding into small intracytoplasmic vesicles (A, B, and C; $\times 95,000$). Bar = 0.1 μ m. The morphology of HIV particles ranged from ring-shaped immature forms to those with single or multiple condensed nucleoids with a characteristic cylinder shape (D-E; $\times 95,000$). Electron-dense, membrane-associated deposits (arrows) were frequently seen in intracytoplasmic vesicles containing numerous virions (F and K; $\times 48,000$).

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1144 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES

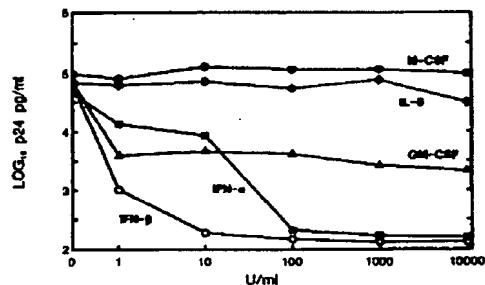


FIGURE 5. Log-log plot of the dose-response curves of anti-HIV treatments. MDM were pretreated for 18 h with the indicated doses of IFN- α , IFN- β , IL-6, GM-CSF, and M-CSF, then infected with HIV (MOI = 0.1), washed, and the treatments re-added. Supernatants were collected for p24 core antigen assay 10 d later, and the \log_{10} of the mean p24 pg/ml of duplicate cultures is shown. Multinucleated giant cells were absent in cultures treated with >1,000 U/ml of IFN- α and >10 U/ml of IFN- β , but were not prevented by IL-6, GM-CSF, and M-CSF. Data for M-CSF from Cetus Corp. are shown, and are essentially identical to data for M-CSF from Genetics Institute.

Cytokine	Dose	\log_{10} mean p24 pg/ml	Cytopathology
IL-1 β U/ml	0	5.04	+
	1	4.98	+
	3	5.00	+
	10	4.59	+
	50	4.04	+
IL-2 U/ml	1	4.80	+
	10	4.85	+
	100	5.05	+
	1,000	4.76	+
TNF- α U/ml	1	4.79	+
	10	4.86	+
	100	4.55	+
	1,000	4.45	+
Lymphotxin (TNF- β) U/ml	1	4.92	+
	10	5.11	+
	100	5.08	+
	1,000	4.86	+
PBMC Control	3%	4.85	+
Supernatant (Percent volume)	10%	4.97	+
	30%	4.80	+
PBMC-Con A	3%	3.32	-
Supernatant (Percent volume)	10%	3.41	-
	30%	3.18	-

MDM were pretreated with the above agents for 18 h, infected with HIV (MOI = 0.1), and washed four times to remove input virus. After replacing the treatment agents, the cultures were incubated for 12 d. p24 core antigen was measured in the culture supernatants and they are reported as the \log_{10} of the mean of duplicate cultures. Cytopathology was scored by the presence or absence of multinucleated giant cells.

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KORNBLUTH ET AL.

1145

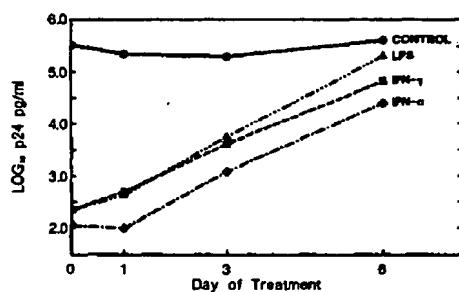


FIGURE 6. Semi-log plot of the time course of effectiveness of anti-HIV treatments. In the same experiment as shown in Fig. 1, MDM were infected with HIV (MOI = 1) and washed four times before adding the treatment, in order to remove p24 core antigen that may have accumulated in the medium. Cultures were treated with IFN- α (5,000 U/ml), IFN- γ (5,000 U/ml), or LPS (10 μ g/ml) at the indicated times after infection with HIV. The final levels of p24 accumulation in the supernatants were measured at intervals throughout the culture period. Only the final p24 levels at 15 d after infection are shown.

ever, these agents were no longer effective in preventing the accumulation of p24 antigen.

Effects of Interferons and LPS on HIV Gene Expression in Macrophages. Although IFN- α , IFN- γ , and LPS suppressed the release of p24 antigen and infectious virus from MDM into their culture media, and eliminated the formation of virions seen by electron microscopy, these techniques did not completely rule out the expression of viral genes inside the cells. Consequently, nucleic acid hybridization was used to evaluate the formation of RNA transcripts and DNA provirus within these cells. Infected MDM that were not treated with cytokines or LPS contained HIV RNA and DNA (Fig. 7). In contrast, IFN- α , IFN- γ , or LPS-treated MDM showed no evidence of viral RNA transcripts or provirus formation. Thus, in the treated cultures, the expression of nonstructural genes and the formation of proviral DNA are also prevented.

Discussion

In this report, IFN- α , IFN- β , IFN- γ , and LPS were shown to protect cultured macrophages from productive infection with a macrophage-tropic strain of HIV-1.

A	B
RNA	DNA
MDM	—
MDM + HIV	—
MDM + HIV + IFN- γ	—
MDM + HIV + IFN- α	—
MDM + HIV + LPS	—

FIGURE 7. Effect of anti-HIV treatments on viral nucleic acids in MDM. MDM cultures (6×10^6 cells per 25-cm² flask) were infected with HIV (MOI = 0.1), washed, and then treated with IFN- γ (1,000 U/ml), IFN- α (1,000 U/ml), or LPS (1 μ g/ml) where indicated. 14 d after infection, supernatants were collected for p24 assay and viral titration. Cellular RNA and DNA were processed as described, slot-blotted onto nitrocellulose, hybridized with a ³²P-HIV-1 probe under stringent conditions, and autoradiographed. (A) Total cellular RNA; (B) undigested DNA. Only untreated MDM contained detectable viral RNA or DNA, and supernatant medium from this culture contained $10^{3.35}$ pg/ml of p24 core antigen and $>10^3$ TCID₅₀/ml of infectious HIV. In contrast, IFN- γ , IFN- α , and LPS-treated MDM did not contain detectable viral RNA or DNA, and supernatant medium from these cultures contained $<10^{2.65}$ pg/ml of p24 core antigen and no detectable infectious HIV.

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1146 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES

Since immunological reactions lead to interferon production, anti-HIV immunity (either natural or induced by a suitable vaccine) may indirectly limit the spread of HIV infection by protecting uninfected cells, thereby augmenting cytotoxic mechanisms for the removal of cells that are already infected. Since several other cytokines do not protect macrophages in this system, the protection afforded by interferons and LPS must be specifically induced.

Interferons are known to inhibit the production of infectious retroviruses by interfering with virus assembly and release (31). Consistent with these late effects, we found that IFN- α , INF- γ , or LPS are effective even when added 3 d after exposure of cells to HIV at a multiplicity of infection of 1 TCID₅₀/cell. This observation suggests that these agents act at a point in the viral life cycle beyond the early events of virus binding, penetration, and uncoating. Interference with reverse transcription and the generation of proviral DNA, the next step in the retroviral replication, are suggested by the absence of detectable viral DNA in the treated macrophages. This might occur by inhibition of viral reverse transcriptase by interferon-induced 2'-5'A oligonucleotides, as reported for the avian myeloblastosis virus enzyme (32). However, this conclusion cannot be supported from the data for two reasons: (a) The method used to detect proviral DNA is not sensitive enough to detect low levels of DNA provirus. (b) The definition of interferon action is best established during a single cycle of viral replication (33); for example, in HIV-infected CEM cells (a T cell leukemia cell line), virus-producing cells are reinfected by their progeny viruses, resulting in the generation of multiple copies of DNA provirus per cell, which correlates with the cytopathic effects of HIV in this cell line (34). By analogy, if macrophages become reinfected by their progeny viruses and if multiple rounds of reinfection are necessary to sustain productive infection, then interferon-induced inhibition of virus release alone would yield data indistinguishable from an effect on reverse transcription. Consequently, further studies are required to clarify the mode of action of interferon and LPS and the fate of HIV upon entry into an interferon- or LPS-treated macrophage.

Further studies are also required to determine if all HIV isolates capable of infecting macrophages are equally susceptible to interferons or LPS inhibition. It is possible that HIV adapts to culture conditions as a result of in vitro mutation and selection pressure. By preparing our viral stock under endotoxin-free conditions, we may have inadvertently selected for an interferon- and LPS-sensitive strain of HIV. We intend to test this possibility using viruses isolated by a different technique (6).

LPS and interferons (especially IFN- γ) are thought to "activate" certain macrophage functions, yet these agents protected MDM in these experiments. We found no evidence that LPS worked indirectly by inducing the secretion of interferon. Although LPS is known to stimulate macrophage production of IL-6 (IFN β 2), IL-6 has no protective effect on its own. Furthermore, no interferon was detectable in the supernatants from LPS-stimulated MDM using a plaque-reduction assay for vesicular stomatitis virus on MDBK cells (data not shown). Whatever the mechanism, these results indicate that inadvertent endotoxin contamination of media and/or serum (21) may confound the results of antiviral studies in macrophages. Endotoxin contamination may contribute to the differences between our results and those recently reported by Koyanagi et al. (35). These authors studied MDM that had been isolated by adherence, exposed to activated complement, detached by EDTA and

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KORNBLUTH ET AL.

1147

scraping, and infected in the presence of the detergent polybrene. Endotoxin levels were not monitored in either the MDM cultures or the PBMC cultures used to grow the viral stocks that they used. In contrast, we have used essentially endotoxin-free conditions and have verified that the MDM were not artificially activated by the culture conditions. The uninfected MDM used in these experiments did not release IL-1 or TNF detected by bioassays, and did not contain detectable mRNA for IL-1, TNF, IL-6, or tissue factor, although LPS readily induced the expression of these genes (data not shown). This pattern of gene expression is consistent with a "resting" or nonactivated macrophage phenotype. By using MDM of defined activation phenotype, an additional element of experimental control is made available. These factors may account for the >10-fold lower level of viral replication in the control cultures of Koyanagi et al. (based on p24 antigen levels) as compared with the data reported here. Suboptimal viral replication may explain the sensitivity of their assay system to viral-enhancing agents, such as colony-stimulating factors. In contrast, our experiments may have used a nearly optimal system for viral replication, accounting for the sensitivity of our assay system to inhibitory agents.

Macrophages are important host cells for lentiviruses such as HIV (1-14) and visna-maedi (36, 37). In sheep, the restricted replication of visna-maedi virus in macrophages has been associated with the production of an unusual immune interferon (36, 37). Interestingly, an unusual type of acid-labile IFN- α has been detected in the serum of HIV-infected individuals (38). A protective function for this type of interferon has not yet been demonstrated, and its presence in HIV infection may actually correlate with a worsened prognosis (39).

Neutralizing antibody to IFN- α has been reported to increase the yield of HIV in PBMC cultures from HIV-infected individuals (40). Reciprocally, the addition of IFN- α or IFN- β , but not IFN- γ , has been reported to suppress the replication of T-lymphotropic strains of HIV in PBMC (41, 42). In cell lines used as models of HIV infection, IFN- α and IFN- γ are similarly active (43-47). However, in HIV-infected individuals *in vivo*, IFN- α does not control non-HIV viral infections effectively (48, 49), and the induction of RNase L activity by IFN-induced 2'-5'A is impaired in PBMC from AIDS patients *in vitro* (50). Consequently, it cannot be assumed that macrophages from HIV-infected individuals will be protected from HIV by interferons as efficiently as macrophages from the uninfected donors used in our experiments. Nevertheless, it is encouraging that macrophages from AIDS patients have an appropriate enhancement of H₂O₂ release after IFN- γ treatment *in vivo* and *in vitro* (51). In this report, we demonstrate the activity of IFN- α , IFN- β , and IFN- γ against a macrophage-tropic strain of HIV-1 using cultured macrophages from normal donors. Since the macrophage may be the first cell type to become infected after exposure to HIV (6, 14), it would be useful to monitor the effects of interferons on monocyte/macrophage infection in clinical trials with these agents.

Summary

To determine the effects of immunomodulatory agents upon HIV replication in macrophages, cultured monocyte-derived macrophages were treated with various substances and then infected with a macrophage-tropic strain of HIV-1. Pretreatment with rIFN- α , IFN- β , and IFN- γ , or bacterial LPS prevented viral replication in macrophages. In treated cultures, little or no infectious HIV or p24 core antigen

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1148 CYTOKINE EFFECTS ON HIV REPLICATION IN MACROPHAGES

was released into the supernatant, no virions were seen by electron microscopy, no viral RNA or DNA was detectable in the cell lysates, and no cytopathology (as determined by multinucleated giant cell formation) occurred. In contrast, pretreatment with a wide dose range of recombinant IL-1 β , IL-2, IL-4, IL-6, M-CSF, TNF, or lymphotoxin failed to protect macrophages from productive infection by HIV. A consistent effect of granulocyte/macrophage-CSF on HIV replication in macrophages was not observed.

In dose response studies, pretreatment with ~100 U/ml of IFN- α , ~10 U/ml of IFN- β , or ~100 U/ml of IFN- γ was sufficient to prevent virion release maximally and to prevent cytopathology completely. In kinetic studies, IFN- α , IFN- γ , or LPS were added to the macrophage cultures either before or after infection with HIV. Even when added 3 d after infection with a multiplicity of 1 50% tissue-culture infectious dose per cell, all three treatments markedly reduced virion release, suggesting that these agents act at a point in the viral life cycle beyond the early events of virus binding, penetration, and uncoating.

These data indicate that HIV replication in previously uninfected macrophages may be regulated by an inducible host cell mechanism. These findings may explain the restricted replication of HIV in macrophages *in vivo* and suggest an antiviral role for interferons in the therapy of HIV infection.

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1149

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1151

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Use of IFN-gamma in patients with AIDS.

Heagy W, Groopman J, Schindler J, Finberg R.

Dana-Farber Cancer Institute, Laboratory of Infectious Diseases, Boston, MA 02115.

The tolerance and toxicity of interferon-gamma (IFN-gamma) was assessed in a phase I/II study of 21 patients with acquired immune deficiency syndrome (AIDS). A highly purified preparation of human recombinant *E. coli*-produced IFN-gamma was given i.v. twice weekly for an 8 week period. Patients were enrolled in the study in groups of four or five; the initial group received an IFN-gamma dose of 0.03 mg/m² and subsequent groups received higher IFN-gamma doses of 0.3, 1, or 3 mg/m². Toxicity resulting from IFN-gamma was minimal and the therapy was well tolerated even at the maximum dose (3 mg/m²). No patients developed antibodies that neutralized IFN-gamma. Clinical responses were observed in 3 of 17 patients with Kaposi's sarcoma (KS). A complete clinical response was achieved in one individual and a partial, temporary regression of KS lesions was observed in two other patients. HIV p24 antigen was decreased in plasma samples obtained from six of nine patients with initially detectable HIV protein. These data suggest that IFN-gamma should be considered as a therapeutic agent, possibly with other antivirals, in the treatment of patients with AIDS.

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A phase I trial of recombinant gamma interferon in patients with cancer.

Foon KA, Sherwin SA, Abrams PG, Stevenson HC, Holmes P, Maluish AE, Oldham RK, Herberman RB.

A total of 11 patients were treated on an escalating, single dose trial of recombinant gamma interferon (rIFN-gamma), 6 patients by the i.m. and 5 patients by the i.v. route of administration. Dose ranges within each individual were from 0.05 mg/m² of IFN (1 mg greater than or equal to 10 X 10⁶ units of IFN) escalating to 10 mg/m². All dosages were delivered twice weekly and the i.v. dose was infused over 5 min. The most common toxicities encountered included fever, chills, fatigue, anorexia, and granulocytopenia. The influenza-like symptoms were very similar to those encountered with IFN-alpha but were generally less severe. The granulocytopenia was dose-related and transient with recovery generally seen within 48-72 h following administration of rIFN-gamma. Absolute granulocyte counts only rarely dropped below 1000/mm³. Hepatotoxicity was not observed. IFN levels were determined by both a bioassay and an enzyme-linked immunosorbent assay. By the i.v. route, the peak level of IFN activity could usually be seen at completion of the infusion with a serum half-life of 30 min. By the i.m. route, the peak level of serum activity was generally detected between 4-8 h with a serum half-life of 4.5 h after the initial elimination phase. Peak IFN levels appeared to correlate with maximum toxicity. One patient with melanoma had a 25% reduction in a cutaneous lesion, but there were no other minimal, partial, or complete responses.

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Recombinant interferon-gamma (immuneron): results of a phase I trial in patients with cancer.

van der Burg M, Edelstein M, Gerlis L, Liang CM, Hirschi M, Dawson A.

Recombinant DNA-produced interferon-gamma (rIFN-gamma) was administered intravenously to patients with solid tumors in a Phase I study. The rIFN gamma was prepared from *Escherichia coli* and purified to greater than 95% with a specific activity of greater than or equal to 30×10^6 units/mg protein. Twenty patients received intravenous bolus injections once weekly for 4 consecutive weeks. They were assigned to one of six dose groups ranging from 1 to 81×10^6 (6) units/m² body surface area; intrapatient dose escalation was not allowed. Patients were monitored intensively for toxicity, but no dose-limiting toxicity was demonstrated. Fever was the predominant side effect, occurring in all patients treated, and usually reached 38-40 degrees C. Short-term somnolence and fatigue were also observed, but no chronic fatigue was seen. Decreases in white blood cell and platelet counts, generally within the normal range, were observed; however, the counts rose again after intervals of 2-5 days. There was no firm evidence of a relationship between adverse effects and dose. No life-threatening side effects were noted and no antibodies developed to either rIFN gamma or *E. coli* proteins. The pharmacokinetics of rIFN gamma did not appear to alter from week 1 to week 4. Calculated half-lives were from 0.8 to 3.5 h. Doses greater than 9×10^6 units/m² gave measurable serum levels for at least 12 h. A partial response of 8 weeks' duration was observed in a patient with hepatoma.

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A preliminary Phase I trial of partially purified interferon-gamma in patients [J Biol Response Mod. 1984]

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Phase I trial of recombinant interferon gamma by 1-hour i.v. infusion.

Vadhan-Raj S, Nathan CF, Sherwin SA, Oettgen HF, Krown SE.

Fifteen patients with advanced malignancy were treated with recombinant interferon gamma (rIFN-gamma) (specific activity approximately 2×10^7 units/mg, purity greater than 99%) given by 1-hour iv infusion three times a week for 6 weeks, at fixed dose levels of 0.1, 0.5, 1.0, or 2.0 mg/m²/day. The common side effects were constitutional symptoms, including fever, chills, myalgias, and headache, but these were less severe than those observed following daily 6-hour iv infusions. Significant changes in blood cell counts and routine serum chemistries were not observed, but there was a dose-dependent increase in serum triglyceride levels. The maximum safely tolerated dose achieved was 1.0 mg/m²/day. Peak serum interferon levels occurred at the midpoint of the infusion and were dose-dependent. rIFN-gamma was rapidly cleared from serum and no detectable activity was found 2 hours after the infusion. Two patients, both with B-cell malignancies, showed objective evidence of tumor regression during the treatment. Treatment was associated with an increase in serum levels of beta 2-microglobulin and the H2O2 secretory capacity of peripheral blood monocytes. We conclude that rIFN-gamma administered by short iv infusion can induce biological activities and causes less toxicity than when given by prolonged iv infusion.

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Phase I trial of combinations of recombinant interferons beta(ser) and gamma in patients with advanced malignancy. [\[Cancer Treat Rep. 1987\]](#)

Recombinant interferon-gamma (immuneron): results of a phase I trial in patients with cancer. [\[J Clin Oncol. 1985\]](#)

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A randomized phase I/II study of continuous versus Intermittent intravenous interferon gamma in patients with metastatic melanoma.

Ernstaff MS, Trautman T, Davis CA, Reich SD, Witman P, Balser J, Rudnick S, Kirkwood JM.

Section of Medical Oncology, Yale School of Medicine, New Haven, CT.

Thirty patients with documented metastatic melanoma were randomly assigned to receive recombinant DNA-produced gamma-interferon (specific activity approximately, 20 MU/mg) intravenously (IV) over either two or 24 hours at dosages of 3, 30, 300, 1,000, or 3,000 micrograms/m². Objective toxicity resembled that of alpha-interferon and included fever, chills, myalgias, headache, and fatigue. Neutropenia, elevations in liver enzymes, tachyarrhythmias, and CNS changes also were noted. Dose-limiting toxicity included neutropenia, liver enzyme abnormality, constitutional symptoms, and a change in mental status. The incidence of toxicity was qualitatively similar in both two- and 24-hour treatment arms, but was quantitatively more severe in the 24-hour continuous infusion arm. Maximum tolerated dose was 1,000 micrograms/m² in both schedules.

Pharmacokinetic studies showed a half-life of six to nine hours. One patient had a complete response after two cycles of therapy and an additional patient entered partial remission after three cycles. Recombinant gamma-interferon (rIFN-gamma) is tolerated at dosages of 1,000 micrograms/m² administered daily either by two or 24 hour infusion for 14 days in patients with metastatic melanoma. The responses documented in this early trial warrant further evaluation for the treatment of metastatic melanoma.

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[Gamma Interferon therapy of cancer patients]

[Article in Japanese]

Kobayashi Y, Urabe A.

3rd Dept. of Internal Medicine, Faculty of Medicine, University of Tokyo.

A phase I and a phase II study of recombinant gamma-interferon (S 6810) were conducted on a cooperative basis involving 11 and 57 institutions, respectively. In the phase I study, a total of 40 courses were administered to 31 patients. High fever exceeding 38 degrees C with chills was observed in approximately 80%. Other toxicities were fatigue (50%), gastrointestinal symptoms (30-40%), changes in hepatic enzymes, and hematological toxicities (20-30%). Dose-limiting factors were judged to be hypotension, leucopenia and CNS toxicity. Since the optimal dose for the phase II study was considered to be 5 X 10(6) U/m2 by daily chronic schedule, a further study was conducted using this dose. Response rates were as follows: 14.3% (renal cell cancer), 11.8% (multiple myeloma) 40.0% (chronic lymphocytic leukemia), 16.7% (non-Hodgkin lymphoma), and 67% (mycosis fungoides). Complete response was obtained in one case each of renal cell cancer, malignant lymphoma and mycosis fungoides. Moreover, intermittent high-dose gamma-interferon against renal cell cancer induced a response rate of 21.4%, significantly higher than the 8.6% obtained by continuous administration. Local injection against cutaneous malignancies resulted in a 55.3% response rate. Anti-viral effect against herpes zoster infection was also preliminarily evaluated. Among 4 cases, 3 responded subjectively well to local injection of gamma-interferon, which is a hopeful result, although a randomized trial is still needed.

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Phase II study of recombinant human interferon gamma (S-6810) on renal cell carcinoma. Summary of two collaborative studies.

Recombinant Human Interferon Gamma (S-6810) Research Group on Renal Cell Carcinoma. [Cancer. 1987](#)

[Phase II study of recombinant human interferon gamma (S-6810) in renal cell carcinoma. Urological Cooperative Study Group of Recombinant Human Interferon Gamma (S-6810). [Gan To Kagaku Ryoho. 1987](#)]

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Recombinant interferon gamma in the treatment of metastatic renal cell carcinoma. Results of a phase II trial.

Otto U, Conrad S, Schneider AW, Klosterhalfen H.

Department of Urology, University of Hamburg, Fed. Rep. of Germany.

A clinical Phase II study was performed to evaluate the efficacy and safety of treatment with interferon gamma in patients with metastatic renal cell carcinoma. Patients received interferon gamma by two different regimens: 1. 100 micrograms/m² 3x/week i.v. over 4 h every other week--low dose. 2. 500 micrograms/m² 5x/week i.v. over 24 h every other week--high dose--for non-responders to regimen 1. The response rate, duration of response, survival and toxicity in the two regimens were evaluated. Treatment with interferon gamma resulted in an overall response rate of 31%, with a duration of response ranging between 2 and 44+ months. Patients responding objectively to interferon gamma or showing stable disease survived significantly longer than non-responders ($p = 0.0056$).

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A phase IA trial of sequential administration recombinant DNA-produced interferons: combination recombinant interferon gamma and recombinant interferon alfa in patients with metastatic renal cell carcinoma. [J Clin Oncol. 1990]

The treatment of metastatic renal cell carcinoma patients with recombinant human gamma interferon. [Cancer J Sci Am. 1998]

Interferon gamma-1b compared with placebo in metastatic renal-cell carcinoma. Canadian Urologic Oncology Group. [N Engl J Med. 1998]

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[Treatment of metastatic kidney cancer with recombinant alpha-2 or gamma interferon. Results of 2 clinical phase II and III studies]

[Article in German]

Otto U, Schneider A, Denkhaus H, Conrad S.

Urologische Universitätsklinik, Hamburg.

In a phase-II and a phase-III study patients with histopathologically documented metastatic renal cell carcinoma were treated either with gamma-interferon in two different doses (100 micrograms/m² 3x/week for 4 h i.v. every other week or 500 micrograms/m² 5x/week for 24 h i.v. every other week) or with alpha-2-interferon alone (18 x 10(6) U 3x/week weekly i.m.) or in combination with vinblastine (0.1 mg/kg every third week i.v.). The purpose of these studies was to evaluate the response rate, the duration of response, the survival, the efficacy and the toxicity of the different forms of treatment. The overall response rate to gamma-interferon was 30% in both regimens. The response rate of treatment with alpha-2-interferon was found to be 31%. The duration of response ranged between 2 and 34+ months in patients treated with gamma-interferon and between 2 and 24+ months in those receiving alpha-2-interferon. Patients with objective tumor response showed a significantly longer survival than those not responding ($p = 0.0056$). Low-dose-gamma-interferon and alpha-2-interferon treatment could be easily done on an outpatient basis. In conclusion, interferon treatment seems to be of value in the therapy of patients with well documented progressive disease in metastatic renal cell cancer.

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EORTC (30885) randomised phase III study with recombinant interferon alpha and recombinant interferon alpha and gamma in patients with advanced renal cell carcinoma. The EORTC Genitourinary Group [Br J Cancer. 1995]

Multiinstitutional home-therapy trial of recombinant human interleukin-2 and interferon alfa-2 in progressive metastatic renal cell cancer [J Clin Oncol. 1995]

Phase II trial of interleukin 2, interferon alpha, and 5-fluorouracil in metastatic renal cell cancer: a cytokine working group study. [J Clin Oncol. 2000]

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Successful treatment of metastatic renal cell carcinoma with a biologically active dose of recombinant interferon-gamma.

Aulitzky W, Gastl G, Aulitzky WE, Herold M, Kemmler J, Mull B, Frick J, Huber C.

Department of Urology, General Hospital, Salzburg, Austria.

We tested the clinical efficacy of a biologically active dose (BAD) of Interferon (IFN)-gamma for treatment of progressive renal cell carcinoma (RCC). Twenty-two RCC patients with disease progression subsequent to nephrectomy were entered on a phase II clinical trial. During an initial dose-finding phase, biochemical responses to repeated once-weekly subcutaneous injections of 10, 100, or 500 micrograms of recombinant IFN-gamma were tested in 16 patients. Results indicated that 100 micrograms IFN-gamma applied once weekly was biologically active with induction of serum beta 2-microglobulin and neopterin. Such a dose induced a nearly maximum response of both markers lasting more than 4 days. This dose was also associated with minimal side effects. A dose of 100 micrograms IFN-gamma given once weekly was, therefore, subsequently given weekly for long-term treatment. During a median time of therapy of 10 months (range, 2 to 32 months) two complete (CR; 20+, 20+ months) and four partial tumor responses (PR; 6+, 7+, 8+, 24+ months) were seen (30% CR plus PR; 95% confidence limits, 12% to 54%) among 20 patients evaluable for response. Patients with refractory disease had significantly lower IFN-gamma-induced increments of serum beta 2-microglobulin than those who achieved clinical remission or stable disease.

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Low-dose gamma-interferon therapy is ineffective in renal cell carcinoma patients with large [\[Eur Urol. 1994\]](#)

Phase II trial of interferon-beta-serine in metastatic renal cell carcinoma. [\[J Clin Oncol. 1990\]](#)

Prolonged interferon-gamma application by subcutaneous infusion in cancer patients: differential response of serum CD14, neopterin, and monocyte HLA class I and II antigens. [\[J Interferon Res. 1992\]](#)

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Phase I-II study of advanced head and neck squamous cell carcinoma patients treated with recombinant human Interferon gamma.

Richtsmeier WJ, Koch WM, McGuire WP, Poole ME, Chang EH.

Department of Otolaryngology-Head Neck Surgery, Johns Hopkins Medical Institutions, Baltimore, Md 21205.

The association of immunodeficiency with head and neck squamous cell carcinoma has generated the concept of supplying immunologically active agents as a means of treating these cancers. One of the most active immunologic messengers is Interferon gamma, which has been observed in our laboratories to also have a direct cytotoxic effect on cultures of squamous cell carcinoma derived from the head and neck. To test the feasibility of treating patients with advanced but resectable head and neck cancer with this agent, we designed a phase I-II trial of recombinant human interferon gamma using a 24-hour infusion repeated weekly for four times. In this study, both tumor and immunologic parameters were studied before and after treatment. Eight patients were entered into the study with the highest recombinant human interferon gamma dose attempted being 0.25 mg/m² per 24 hours. Minimal side effects were observed. Three patients had clinically measurable responses, four had stabilization of disease, and one had progression while receiving treatment. Histopathologic results of treatment were similar to in vitro observations. Necrosis, as well as differentiation of tumor cells, was observed. In some tumors there was a marked decrease in cellularity without a change in tumor volume due to increased extracellular keratin deposition. Our study indicates that evaluation of adoptive immunotherapy trials in head and neck cancer needs to include parameters other than simple tumor regression as an end point, otherwise therapeutically important lymphokine-induced changes may be missed. Further evaluation of recombinant human interferon gamma and agents that induce human interferon gamma are warranted.

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Combination immunotherapy of squamous cell carcinoma of the head and neck [Am J Clin Oncol. 2000]

Augmentation of the lymphokine-activated killer cell response in head and neck cancer patients by combination interleukin-2 and interferon-alpha. [Am J Surg. 1991]

Combined interferon-alfa, 13-cis-retinoic acid, and alpha-tocopherol in locally advanced head and neck squamous cell carcinoma: novel bioadjuvant phase I [J Clin Oncol. 2001]

Combination nonviral interleukin-2 gene immunotherapy for head and neck cancer: from bench top to bedside. [Laryngoscope. 2005]

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[Long-term results in the treatment of metastasizing kidney carcinoma using recombinant alpha-2 or gamma interferon]

[Article in German]

Schneider AW, Otto U, Conrad S, Klosterhalfen H.

Urologische Universitätsklinik und Poliklinik, Hamburg.

To evaluate whether response to alpha-2- or gamma-interferon in metastatic renal cell carcinoma is associated with a prolonged survival, we studied a total of 65 patients being treated in two phase-II- or phase-III-trials between 1984 and 1986 with one of these interferons. After a median follow-up of 3 years for the alpha-interferon-treated patients and of more than 4 years for gamma-interferon-therapy, respectively, there is a significantly increased duration of survival for patients showing an objective response or stabilisation of the disease due to either therapy compared to those with continuing progression. Therapy with alpha-interferon leading to objective remissions in 26% or with gamma-interferon with an objective response rate of 36% therefore is beneficial for responding patients and can be performed with moderate side effects on an out-patient basis.

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A randomized phase II trial comparing two different sequence combinations of autologous vaccine and human recombinant interferon gamma and human recombinant interferon alpha2B therapy in patients with metastatic renal cell carcinoma: clinical outcome and analysis of immunological parameters. [\[J Urol. 2000\]](#)

Interferon gamma-1b compared with placebo in metastatic renal-cell carcinoma. Canadian Urologic Oncology Group. [\[N Engl J Med. 1998\]](#)

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Clinical study with recombinant interferon gamma versus interferon alpha-2c in patients with condylomata acuminata.

Reichel RP, Fitz R, Neumann R, Pohl-Markl H, Pichler E, Hoffer Z, Budiman R.

1st Department of Gynaecology and Obstetrics, University of Vienna, Austria.

A multi-centre, randomized, open-label trial was conducted to evaluate the safety and efficacy of recombinant interferon (rIFN) alpha-2c versus rIFN gamma in patients with recurrent or persistent condylomata acuminata (CA). Thirty-three such patients were treated either with 6 micrograms rIFN alpha-2c or with 0.1 mg rIFN gamma (both equivalent to 2 x 10E6 IU), single dose, subcutaneously 3 times a week for 6 weeks. In case of no complete clearance at week 10, a second course of treatment with the other type of rIFN was given. There was no significant difference in the complete clearance proportions at week 10 between the two treatment groups (3/16 vs 6/17). No relapses occurred in these patients during the 16 weeks' follow-up. Further clearances during the follow-up resulted in a total complete clearance proportion of 14/33 at the end of study. The treatment was well tolerated. Repeated interferon therapy has its place in treating persistent or recurrent condylomas.

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Recurrent condylomata acuminata treated with recombinant interferon alfa-2a. A multicenter double-blind placebo-controlled clinical trial. Condylomata International Collaborative Study Group [AMA. 1991]

A phase IIA trial of sequential administration recombinant DNA-produced interferons: combination recombinant interferon gamma and recombinant interferon alfa in patients with metastatic renal cell carcinoma. [J Clin Oncol. 1990]

Recurrent condylomata acuminata treated with recombinant interferon alpha-2a. A multicenter double-blind placebo-controlled clinical trial. Condylomata International Collaborative Study Group [AMA. 1991]

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Phase II trial of low dose gamma-interferon in metastatic renal cell carcinoma.

Ellerhorst JA, Kilbourn RG, Amato RJ, Zukiwski AA, Jones E, Logothetis CJ.

Department of Genitourinary Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston 77030.

We conducted a phase II trial to confirm the activity of fixed, low dose gamma-interferon in metastatic renal cell carcinoma. A total of 35 patients with metastatic renal cell carcinoma, who had not received prior immunotherapy and who had a Zubrod performance status of 2 or less, was enrolled in this study. Primary tumors were controlled by nephrectomy or embolization before treatment began. gamma-Interferon was administered weekly as a subcutaneous injection at a fixed dose of 100 micrograms. Toxic effects were limited to low grade fever, chills and myalgias within 24 hours of injection. There were no incidences of grade 3 or 4 toxicity. Responses could be evaluated in 34 patients. There were 1 complete and 4 partial responses, for an objective response rate of 15% (95% confidence interval 5 to 32%). Durations of response to date are 21+, 17+, 13+, 9 and 2 months. We conclude that gamma-interferon is an active agent for metastatic renal cell carcinoma when administered according to this dose and schedule. The response rate compares favorably with those of alpha-interferon and interleukin-2, and toxicity is minimal. gamma-Interferon has excellent potential for use in combination with other biological or chemotherapeutic agents and in the adjuvant setting.

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Daily alternating administration of high-dose alpha-2b-interferon and interleukin-2 bolus infusion in metastatic renal cell cancer. A phase II study. [Cancer. 1993]

The treatment of metastatic renal cell carcinoma patients with recombinant human gamma interferon. [Cancer J Sci Am. 1998]

Stratification by risk factors predicts survival on the active treatment arm in a randomized phase II study of interferon-gamma plus/minus interferon-alpha in advanced renal cell carcinoma (E6890) [Jad Oncol. 2003]

[Results of low dosage cyclic interferon-gamma therapy of metastatic renal cell [Cancer. 1993]

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[J Clin Oncol](#)

Intraperitoneal recombinant interferon gamma in ovarian cancer patients with residual disease at second-look laparotomy.

Pujade-Lauraine E, Guastalla JP, Colombo N, Devillier P, Francois E, Fumoleau P, Monnier A, Nooy M, Mignot L, Bugat R, Marques C, Mousseau M, Netter G, Maloisel F, Larbaoui S, Brandely M.

Hotel Dieu Hospital, Paris, France.

PURPOSE: The purpose of this study was to evaluate the efficacy and tolerance of recombinant human interferon gamma (rIFN-gamma) as second-line treatment in patients with persistent disease at second-look laparotomy. **PATIENTS AND METHODS:** One hundred eight patients with residual disease at second-look laparotomy were treated with rIFN-gamma (20 x 10⁶ IU/m²) administered intraperitoneally (IP) twice a week for 3 to 4 months. In the absence of clinically assessable disease, response to rIFN-gamma was assessed with a third-look laparotomy. **RESULTS:** Of 98 assessable patients, 31 (32%) achieved a surgically documented response, including 23 patients (23%) with a complete response (CR). The age and size of residual tumor were significant prognostic factors for the response to rIFN-gamma. A 41% CR rate was observed in 41 patients younger than 60 years and with residual tumor less than 2 cm. The probability of response was independent of previous response to first-line chemotherapy. The median duration of response was 20 months and the 3-year survival rate in responders was 62%. Response to rIFN-gamma was the most significant prognostic factor for survival of patients with residual disease. Adverse events included fever, flu-like syndrome, neutropenia, and liver enzyme disturbances. No significant peritoneal fibrosis was noted. **CONCLUSION:** These results support the potential interest of IP rIFN-gamma as adjuvant treatment in ovarian cancer. Controlled prospective trials are required to determine its place in the therapeutic strategy of this malignancy.

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